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TWO-HYBRID ASSAY THAT DETECTS
HIV-1 REVERSE TRANSCRIPTASE DIMERIZATION

This invention is a continuation-in-part and claims the benefit of U.S. Serial No. 09/588,939, filed June 6, 2000, the contents of which are hereby incorporated by reference into this application.

The invention disclosed herein was made in part with Government support under NIH Grant No. AI 27690. Accordingly, the government has certain rights in this invention.

Throughout this application, various publications are referenced within parentheses. Disclosures of these publications in their entirety are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citations for these references may be found immediately preceding the claims.

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BACKGROUND OF THE INVENTION

HIV-1 reverse transcriptase (RT) catalyzes the conversion of genomic RNA into cDNA. The enzyme is a heterodimer of p66 and p51 subunits, and the dimerization of these subunits is required for optimal enzyme activity. To analyze this process at the genetic level we developed constructs that permit the detection of the interaction between these subunits in the

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yeast two-hybrid system. Genetic analysis of RT subdomains required for heterodimerization revealed that the fingers and palm of p66 were dispensable for p51 interaction. However, as little as a 26-amino acid deletion at the C terminus of p51 prevented dimerization with p66. A primer grip mutation, L234A, previously shown to inhibit RT dimerization by biochemical assays, also prevented RT dimerization in the yeast two-hybrid system. Second-site mutations that restored RT dimerization in yeast to the L234A parent were recovered in the tryptophan repeat region at the dimer interface and at the polymerase active site, suggesting the involvement of these sites in RT dimerization. In vitro binding experiments confirmed the effects of the L234A mutation and the suppressor mutations on the interaction of the two subunits. The RT two-hybrid assay should facilitate the extensive genetic analysis of RT dimerization and should make possible the rapid screening of potential inhibitors of this essential process.

The HIV type 1 (HIV-1) reverse transcriptase (RT) is required for the conversion of genomic RNA into double-stranded proviral DNA, catalyzed by the RNA- and DNA-dependent polymerase and ribonuclease H activities of the enzyme. HIV-1 RT is an asymmetric dimer formed by the association of p66 and p51 polypeptides, which are cleaved from a large Pr160^{GagPol} precursor by the viral protease during virion assembly. p51 contains identical N-terminal sequences as p66, but lacks the C-terminal ribonuclease H (RNase H) domain (1). The structure of HIV-1 RT has been elucidated by x-ray crystallography in a variety of configurations, including

unliganded (2), complexed to nonnucleoside RT inhibitors (3),
 or complexed with double-stranded DNA either with (4) or
 without deoxynucleotide triphosphate (5, 6). Such analyses
 have shown that p66 can be divided structurally into the
 5 polymerase and RNase H domains, with the polymerase domain
 further divided into the fingers, palm, thumb and connections
 subdomains (6). Although p51 has the same polymerase domains
 as p66, the relative orientations of these individual domains
 differ markedly, resulting in p51 assuming a closed
 10 structure.

The RT heterodimer represents the biologically relevant form
 of the enzyme; the monomeric subunits have only low catalytic
 activity (7). Structural analysis reveals three major
 15 contacts between p66 and p51, with most of the interaction
 surfaces being largely hydrophobic (8, 9). The three
 contacts comprise an extensive dimer interface that includes
 the fingers subdomain of p51 with the palm of p66, the
 connection subdomains of both subunits, and the thumb
 20 subdomain of p51 with the RNase H domain of p66 (9).

Several single amino acid substitutions in HIV-1 RT have been
 shown to inhibit heterodimer association (10-12). These
 include the mutations L234A (10,11), G231A (11) and W229A
 25 (11), all located in the primer grip region of the p66
 subunit, and L289K (12) in the thumb subdomain. Remarkably,
 these mutations are not located at the dimer interface and
 probably mediate their effects indirectly through
 conformational changes in the p66 subunit.

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Several biochemical assays have been used previously to specifically measure RT dimerization. Some are based on the physical separation of monomers and dimers as determined by analytical ultracentrifugation (8) and gel filtration (7).
5 Other assays include intrinsic tryptophan fluorescence (13), chemical crosslinking (14), the use of affinity tags (15) and polymerase activity itself (7). Although these methods detect dimerization, they either lack specificity or are not easy to perform. Moreover, these assays do not facilitate
10 the rapid genetic analysis of protein-protein interactions under physiological conditions nor are they suitable for high throughput screening for RT dimerization inhibitors.

The yeast two-hybrid (Y2H) system (16) has been exploited to
15 study the homomeric interactions of several retroviral proteins (see, e.g., ref. 17), and heteromeric interactions between viral proteins and various cellular partners (see, e.g., ref. 18). We have used this system to perform a genetic analysis of the determinants of RT dimerization. In
20 addition, we have identified second-site mutations that restore heterodimerization to a noninteracting mutant p66.

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- reporter gene which is activated in the presence of a complex between the p66 subunit polypeptide and the p51 subunit polypeptide, and determining the level of activity of the reporter gene in the cell in the presence of the compound; and
- 5 b) comparing the level of activity of the reporter gene determined in step (a) with a level of activity of the reporter gene determined in the absence of the compound, wherein an increased level of activity of the reporter
- 10 gene determined in step (a) indicates that the compound is an activator of the formation of the complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and the p66 subunit polypeptide of HIV-1 reverse transcriptase, thereby indicating that the
- 15 compound inhibits HIV-1 reverse transcriptase.

This invention provides a method of determining whether a compound enhances formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51

20 subunit polypeptide of HIV-1 reverse transcriptase which comprises:

- a) contacting a yeast cell with the compound, which cell comprises (i) a first plasmid which expresses a fusion protein comprising a p66 subunit polypeptide of HIV-1
- 25 reverse transcriptase, (ii) a second plasmid which expresses a fusion protein comprising a p51 subunit polypeptide of HIV-1 reverse transcriptase, and (iii) a reporter gene which is activated in the presence of a complex between the p66 subunit polypeptide and the p51
- 30 subunit polypeptide, and determining the level of

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activity of the reporter gene in the cell in the presence of the compound; and

- b) comparing the level of activity of the reporter gene determined in step (a) with a level of activity of the reporter gene determined in the absence of the compound, wherein an increased level of activity of the reporter gene determined in step (a) indicates that the compound is an activator of the formation of the complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and the p66 subunit polypeptide of HIV-1 reverse transcriptase.

This invention provides a method of determining whether a compound inhibits HIV-1 reverse transcriptase which comprises:

- a) contacting a yeast cell with the compound, which cell comprises (i) a first plasmid which expresses a fusion protein comprising a first p66 subunit polypeptide of HIV-1 reverse transcriptase, (ii) a second plasmid which expresses a fusion protein comprising a second p66 subunit polypeptide of HIV-1 reverse transcriptase, and (iii) a reporter gene which is activated in the presence of a complex between the first p66 subunit polypeptide and the second p66 subunit polypeptide, and determining the level of activity of the reporter gene in the cell in the presence of the compound; and
- b) comparing the level of activity of the reporter gene determined in step (a) with a level of activity of the reporter gene determined in the absence of the compound, wherein a decreased level of activity of the reporter

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second p66 subunit polypeptide of HIV-1 reverse transcriptase.

This invention provides a method of determining whether a
5 compound inhibits HIV-1 reverse transcriptase which comprises:

- a) contacting a yeast cell with the compound, which cell comprises (i) a first plasmid which expresses a fusion protein comprising a first p66 subunit polypeptide of
10 HIV-1 reverse transcriptase, (ii) a second plasmid which expresses a fusion protein comprising a second p66 subunit polypeptide of HIV-1 reverse transcriptase, and (iii) a reporter gene which is activated in the presence of a complex between the first p66 subunit polypeptide
15 and the second p66 subunit polypeptide, and determining the level of activity of the reporter gene in the cell in the presence of the compound; and
- b) comparing the level of activity of the reporter gene determined in step (a) with a level of activity of the
20 reporter gene determined in the absence of the compound, wherein an increased level of activity of the reporter gene in step (a) indicates that the compound is an activator of the formation of the complex between the first p66 subunit polypeptide of HIV-1 reverse
25 transcriptase and the second p66 subunit polypeptide of HIV-1 reverse transcriptase, thereby indicating that the compound inhibits HIV-1 reverse transcriptase.

This invention provides a method of determining whether a
30 compound enhances formation of a complex between a first p66

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HIV-1 reverse transcriptase; and

- c) admixing the compound with a pharmaceutically acceptable carrier.

5 This invention provides a method of inhibiting formation of a complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase, which comprises contacting either (1) the p51 subunit polypeptide, (2) the p66 subunit polypeptide,
10 or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of a compound determined to do so by the method of claim 2, so to thereby inhibit formation of a complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit
15 polypeptide of HIV-1 reverse transcriptase.

This invention provides a method of enhancing formation of a complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse
20 transcriptase, which comprises contacting either (1) the p51 subunit polypeptide, (2) the p66 subunit polypeptide, or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of a compound determined to do so by the method of claim 4, so to thereby
25 enhance formation of a complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase.

This invention provides a method of inhibiting formation of
30 a complex between a first p66 subunit polypeptide of HIV-1

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reverse transcriptase and a second p66 subunit polypeptide of HIV-1 reverse transcriptase, which comprises contacting either (1) the first p66 subunit polypeptide, (2) the second p66 subunit polypeptide, or (3) both the first p66 subunit polypeptide and the second p66 subunit polypeptide, with an effective amount of a compound determined to do so by the method of claim 6, so to thereby inhibit formation of a complex between the first p66 subunit polypeptide of HIV-1 reverse transcriptase and the second p66 subunit polypeptide of HIV-1 reverse transcriptase.

This invention provides a method of enhancing formation of a complex between a first p66 subunit polypeptide of HIV-1 reverse transcriptase and a second p66 subunit polypeptide of HIV-1 reverse transcriptase, which comprises contacting either (1) the first p66 subunit polypeptide, (2) the second p66 subunit polypeptide, or (3) both the first p66 subunit polypeptide and the second p66 subunit polypeptide, with an effective amount of a compound determined to do so by the method of claim 8, so to thereby enhance formation of a complex between the first p66 subunit polypeptide of HIV-1 reverse transcriptase and the second p66 subunit polypeptide of HIV-1 reverse transcriptase.

This invention provides a compound determined to be capable of inhibiting formation of a complex between a p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase.

This invention provides a compound determined to be capable

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of enhancing formation of a complex between a p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase.

5 This invention provides a compound determined to be capable of inhibiting formation of a complex between a first p66 subunit polypeptide of HIV-1 reverse transcriptase and a second p66 subunit polypeptide of HIV-1 reverse transcriptase.

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This invention provides a compound determined to be capable of enhancing formation of a complex between a first p66 subunit polypeptide of HIV-1 reverse transcriptase and a second p66 subunit polypeptide of HIV-1 reverse transcriptase.

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BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1

RT fusion constructs, encoded fusion proteins and expression
 5 of fusions in yeast reporter strains. The six-alanine linker
 is denoted by the hatched box, and the HA epitope by black
 shaded regions. p66 and p51 indicate the 66 kDa and 51 kDa
 subunits of HIV-1 RT, respectively. Expression of fusion
 proteins was determined by introducing the indicated plasmids
 10 into CTY10-5d, except for p66GBT9 and p51AS2-1 which were
 introduced into HF7c. Fusion protein expression was detected
 by probing yeast protein lysates with anti-RT antibodies as
 described in the Materials and Methods. ++, high; +,
 moderate; +/-, low and -, undetectable protein expression.
 15 ND, not done.

FIGURE 2

Interaction of p66 deletion mutants with Gal4AD-HA-51 fusion
 protein. p66 polymerase domains were fused to the C-terminus
 20 of lexA87 in pSH2-1. CTY10-5d was cotransformed with the
 appropriate constructs. Transformants were lifted onto
 nitrocellulose and subjected to β -gal colony lift assay to
 determine intensities of blue color produced as defined in
 Tables 1 and 2. β -gal activity from liquid assays is
 25 expressed in Miller Units. Expression in CTY10-5d of p66
 fusion proteins was detected using anti-lexA polyclonal
 antibodies. Expression levels are as defined as in the
 legend for Fig. 1.

30 FIGURE 3

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Interaction of C-terminal deletion mutants of p51 with
lexA202-Ala-66. p51 domains were fused to the C-terminus of
the Gal4AD in pACTII. Deletions at the C-terminus are
denoted by the number of amino acids missing from the end of
5 p51. β -gal activity was determined as described in the
legend of Fig. 2. Expression of p51 fusion proteins in
CTY10-5d was detected using anti-GAL4AD antibodies, and
expression levels are as denoted in the legend for Fig. 1.

10 **FIGURE 4**

L234A inhibits RT dimerization in the Y2H assay. CTY10-5d
was cotransformed with expression constructs, and yeast
patches were subjected to both the β -gal colony lift and
liquid assays. The green is hydrolyzed X-gal and reflects
15 β -gal activity. p66wt and p51wt denote wild-type
lex202-Ala-HX66 and Gal4AD-HX51 fusion proteins,
respectively. pAD denotes pGADNOT. p66mut and p51mut denote
RT fusion proteins lex202-Ala-66-L234A and Gal4AD-51-L234A,
respectively.

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FIGURE 5

Ribbon diagram of unliganded HIV-1 RT showing position of
L234A primer grip mutation and locations of suppressors
(shaded black). The figure was generated by MOLSCRIPT (38)
25 and RASTER3D (39) with coordinates (2) retrieved from the
Research Collaboratory for Structural Bioinformatics (RCSB)
Protein Data Bank (PDB) (<http://www.rcsb.org/pdb>, PDB ID:
1HMY.pdb). Domains are defined as in (3) with fingers, blue;
palm, green; thumb, yellow; connection, red; and RNase H in
30 purple. Domains in p66 are in fully saturated colors,

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whereas in p51 they have decreased saturation. Secondary structure was assigned using DSSP (40). Spirals represent alpha-helices, arrows denote beta-strands.

5 **FIGURE 6**

In vitro assay for binding of GST-p51 and p66 to form active RT heterodimers. Panel A: Bacterial lysates containing GST-p51 and various p66 proteins as indicated were incubated overnight and captured on glutathione beads. The complexes
10 were eluted, resolved by SDS-PAGE, blotted to membrane and detected by monoclonal anti-RT antibodies. Mock, GST-p51 alone. Panel B: An aliquot of each incubation mix, reflecting input protein, was directly analyzed by SDS-PAGE and Western blot as in Panel A. Panel C: Bound proteins were eluted with
15 glutathione and assayed for RT activity with homopolymeric template-primer. Values are normalized to the wild-type control.

FIGURE 7

20 Dose-response curve showing the enhancement by NNRTIs of b-gal activity in yeast cotransformed with lexA₈₇₋₆₆ and Gal4AD-51. The fold increase in b-gal activity was calculated by dividing b-gal activity (in Miller Units) for each drug concentration with the b-gal activity from cells grown in the
25 absence of inhibitor. The data represents the average results from two independent experiments. The concentration of drug that mediates a 5-fold increase in b-gal activity is shown in parenthesis. A: b-gal enhancement activity of the NNRTIs, efavirenz, HBY 097, a-APA, nevirapine, 8-Cl-TIBO and
30 delavirdine. B: b-gal enhancement activity of the

carboxanilide class of NNRTIs.

Figure 8

Effect of the Y181C mutation on enhancement of b-gal activity
5 in yeast by nevirapine. Yeast expressing wild-type lexA_{87-66}
and Gal4AD-51 or mutant $\text{lexA}_{87-66}\text{Y181C}$ and wild type Gal4AD-51
were grown in the presence of nevirapine and assayed for b-
gal activity. Results are expressed as fold increase in b-
gal activity compared to untreated cells. Values on top of
10 each bar indicates b-gal activity (in Miller Units) +/-
standard deviation.

Figure 9

Effect of efavirenz on b-gal activity in yeast expressing the
15 dimerization defective mutants L234A and W401A. Yeast
expressing wild-type p66 bait and p51 prey fusions, mutant
p66 bait and wild-type p51 prey and mutant p66 bait and
mutant p51 prey fusions were assayed for b-gal activity.
Results are expressed as the fold increase in b-gal activity
20 compared to untreated controls. Values on top of each bar
indicates b-gal activity in Miller Units. Effect of efavirenz
on yeast expressing bait and prey fusions with the W401A
change (A) or L234A change (B).

25 Figure 10

Coimmunoprecipitation assay detecting heterodimer formation in
yeast propagated in the presence of NNRTIs. (A): Yeast
expressing p66 bait and p51 prey fusions containing the W401A
mutation were grown in the presence of efavirenz (EFV), UC781
30 or no drug. After growth, yeast were processed in the

Figure 13

Western blot analysis of heterodimer formation after pretreatment of one of the subunits with efavirenz. p66-His, GST-p51 and M15 bacterial lysate were preincubated in the absence or presence of 10 - 1000 fold molar excess of efavirenz. Lysates were washed and the presence of residual efavirenz was assayed by the addition of GST-p51, p66-His or both subunits, respectively. Heterodimers were captured and detected as described in the legend of Fig. 11.

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Figure 14

Molecular surface representation of the p66 and p51 subunits of HIV-1 RT. Residues colored yellow (p66) or magenta (p51) are amino acids that are not accessible to solvent in the presence of the other subunit in the heterodimeric form. The NNRTI binding pocket is shown in red. The sum of the surface areas colored in yellow and magenta is the total buried surface area at the interface of the two subunits.

15

Figure 15

Binding of delavirdine (BHAP) (A) and UC781 (B) at the interface of the p66 (magenta) and p51 (yellow) subunits of the HIV-1 RT. Delavirdine, a large inhibitor, is bound further away from the p66/p51 interface. The relative orientation of the inhibitors in the NNRTI binding pocket is shown in (C). Some residues that comprise the NNRTI binding site have been omitted for clarity.

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DETAILED DESCRIPTION OF THE INVENTION

This invention provides a method of determining whether a compound inhibits HIV-1 reverse transcriptase which
5 comprises:

- a) contacting a yeast cell with the compound, which cell comprises (i) a first plasmid which expresses a fusion protein comprising a p66 subunit polypeptide of HIV-1 reverse transcriptase, (ii) a second plasmid which
10 expresses a fusion protein comprising a p51 subunit polypeptide of HIV-1 reverse transcriptase, and (iii) a reporter gene which is activated in the presence of a complex between the p66 subunit polypeptide and the p51 subunit polypeptide, and determining the level of
15 activity of the reporter gene in the cell in the presence of the compound; and
- b) comparing the level of activity of the reporter gene determined in step (a) with a level of activity of the reporter gene determined in the absence of the compound,
20 wherein a decreased level of activity of the reporter gene in step (a) indicates that the compound inhibits formation of a complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and the p66 subunit polypeptide of HIV-1 reverse transcriptase,
25 thereby indicating that the compound inhibits HIV-1 reverse transcriptase.

This invention provides a method of determining whether a compound inhibits formation of a complex between a p66
30 subunit polypeptide of HIV-1 reverse transcriptase and a p51

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subunit polypeptide of HIV-1 reverse transcriptase which comprises:

- 5 a) contacting a yeast cell with the compound, which cell comprises (i) a first plasmid which expresses a fusion protein comprising a p66 subunit polypeptide of HIV-1 reverse transcriptase, (ii) a second plasmid which expresses a fusion protein comprising a p51 subunit polypeptide of HIV-1 reverse transcriptase, and (iii) a
10 reporter gene which is activated in the presence of a complex between the p66 subunit polypeptide and the p51 subunit polypeptide, and determining the level of activity of the reporter gene in the cell in the presence of the compound; and
- 15 b) comparing the level of activity of the reporter gene determined in step (a) with a level of activity of the reporter gene determined in the absence of the compound, wherein a decreased level of activity of the reporter gene in step (a) indicates that the compound inhibits
20 formation of a complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and the p66 subunit polypeptide of HIV-1 reverse transcriptase.

This invention provides a method of determining whether a
25 compound inhibits HIV-1 reverse transcriptase which comprises:

- 30 a) contacting a yeast cell with the compound, which cell comprises (i) a first plasmid which expresses a fusion protein comprising a p66 subunit polypeptide of HIV-1 reverse transcriptase, (ii) a second plasmid which

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- expresses a fusion protein comprising a p51 subunit polypeptide of HIV-1 reverse transcriptase, and (iii) a reporter gene which is activated in the presence of a complex between the p66 subunit polypeptide and the p51 subunit polypeptide, and determining the level of activity of the reporter gene in the cell in the presence of the compound; and
- 5 b) comparing the level of activity of the reporter gene determined in step (a) with a level of activity of the reporter gene determined in the absence of the compound, wherein an increased level of activity of the reporter gene determined in step (a) indicates that the compound is an activator of the formation of the complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and the p66 subunit polypeptide of HIV-1 reverse transcriptase, thereby indicating that the compound inhibits HIV-1 reverse transcriptase.
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This invention provides a method of determining whether a compound enhances formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase which comprises:

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- a) contacting a yeast cell with the compound, which cell comprises (i) a first plasmid which expresses a fusion protein comprising a p66 subunit polypeptide of HIV-1 reverse transcriptase, (ii) a second plasmid which expresses a fusion protein comprising a p51 subunit polypeptide of HIV-1 reverse transcriptase, and (iii) a reporter gene which is activated in the presence of a
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reporter gene determined in the absence of the compound, wherein a decreased level of activity of the reporter gene in step (a) indicates that the compound inhibits formation of a complex between the first p66 subunit polypeptide of HIV-1 reverse transcriptase and the
5 second p66 subunit polypeptide of HIV-1 reverse transcriptase, thereby indicating that the compound inhibits HIV-1 reverse transcriptase.

10 This invention provides a method of determining whether a compound inhibits formation of a complex between a first p66 subunit polypeptide of HIV-1 reverse transcriptase and a second p66 subunit polypeptide of HIV-1 reverse transcriptase which comprises:

- 15 a) contacting a yeast cell with the compound, which cell comprises (i) a first plasmid which expresses a fusion protein comprising a first p66 subunit polypeptide of HIV-1 reverse transcriptase, (ii) a second plasmid which expresses a fusion protein comprising a second p66
20 subunit polypeptide of HIV-1 reverse transcriptase, and (iii) a reporter gene which is activated in the presence of a complex between the first p66 subunit polypeptide and the second p66 subunit polypeptide, and determining the level of activity of the reporter gene in the cell
25 in the presence of the compound; and
- b) comparing the level of activity of the reporter gene determined in step (a) with a level of activity of the reporter gene determined in the absence of the compound, wherein a decreased level of activity of the reporter
30 gene in step (a) indicates that the compound inhibits

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formation of a complex between the first p66 subunit polypeptide of HIV-1 reverse transcriptase and the second p66 subunit polypeptide of HIV-1 reverse transcriptase.

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This invention provides a method of determining whether a compound inhibits HIV-1 reverse transcriptase which comprises:

- 10 a) contacting a yeast cell with the compound, which cell comprises (i) a first plasmid which expresses a fusion protein comprising a first p66 subunit polypeptide of HIV-1 reverse transcriptase, (ii) a second plasmid which expresses a fusion protein comprising a second p66 subunit polypeptide of HIV-1 reverse transcriptase, and
15 (iii) a reporter gene which is activated in the presence of a complex between the first p66 subunit polypeptide and the second p66 subunit polypeptide, and determining the level of activity of the reporter gene in the cell in the presence of the compound; and
- 20 b) comparing the level of activity of the reporter gene determined in step (a) with a level of activity of the reporter gene determined in the absence of the compound, wherein an increased level of activity of the reporter gene in step (a) indicates that the compound is an
25 activator of the formation of the complex between the first p66 subunit polypeptide of HIV-1 reverse transcriptase and the second p66 subunit polypeptide of HIV-1 reverse transcriptase, thereby indicating that the compound inhibits HIV-1 reverse transcriptase.

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This invention provides a method of determining whether a compound enhances formation of a complex between a first p66 subunit polypeptide of HIV-1 reverse transcriptase and a second p66 subunit polypeptide of HIV-1 reverse transcriptase which comprises:

- a) contacting a yeast cell with the compound, which cell comprises (i) a first plasmid which expresses a fusion protein comprising a first p66 subunit polypeptide of HIV-1 reverse transcriptase, (ii) a second plasmid which expresses a fusion protein comprising a second p66 subunit polypeptide of HIV-1 reverse transcriptase, and (iii) a reporter gene which is activated in the presence of a complex between the first p66 subunit polypeptide and the second p66 subunit polypeptide, and determining the level of activity of the reporter gene in the cell in the presence of the compound; and
- b) comparing the level of activity of the reporter gene determined in step (a) with a level of activity of the reporter gene determined in the absence of the compound, wherein an increased level of activity of the reporter gene in step (a) indicates that the compound is an activator of the formation of the complex between the first p66 subunit polypeptide of HIV-1 reverse transcriptase and the second p66 subunit polypeptide of HIV-1 reverse transcriptase, thereby indicating that the compound inhibits HIV-1 reverse transcriptase.

The methods described herein may also be adapted to other types of cells in addition to a yeast cell. Other cell types include but are not limited to eucaryotic, procaryotic,

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bacteria, E. coli, mammalian and human cells.

In one embodiment of the methods described herein, (a) the fusion protein expressed by the first plasmid comprises a peptide having a DNA binding domain, and (b) the fusion protein expressed by the second plasmid comprises a peptide having a transcription activation domain.

In one embodiment of the methods described herein, (a) the fusion protein expressed by the first plasmid comprises a peptide having a transcription activation domain, and (b) the fusion protein expressed by the second plasmid comprises a peptide having a DNA binding domain.

In one embodiment of the fusion proteins described herein, the peptide having a DNA binding domain is N-terminal relative to the p66 or p51 subunit polypeptide. In another embodiment, the peptide having a DNA binding domain is C-terminal relative to the 51 or p66 subunit polypeptide. The peptide having a DNA binding domain may be bound in the fusion protein to the p51 or p66 subunit polypeptides. In one embodiment, they are bound by peptide bonds. Alternatively, the fusion protein may also comprise one or more additional components, such as a peptide linker and/or an epitope tag. These additional components may separate the peptides from the p51 or p66 subunit polypeptide. The various components may be bound to each other by peptide bonds.

In one embodiment of the fusion proteins described herein, the peptide having a transcription activation domain is N-

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In one embodiment of the fusion proteins described herein, the DNA binding domain is a LexA DNA binding domain. The amino acid and nucleic acid sequences for LexA may be found
5 at Genbank Accession No. J01643. In one embodiment of the methods described herein, the peptide having a DNA binding domain comprises LexA amino acid residues 1-87. The portion of LexA which corresponds to amino acid residues 1-87 may comprise a LexA DNA binding domain. In one embodiment of the
10 methods described herein, the peptide having a DNA binding domain comprises LexA amino acid residues 1-202. The portion of LexA which corresponds to amino acid residues 1-202 may comprise a LexA DNA binding domain.

15 In one embodiment of the fusion proteins described herein, the DNA binding domain is a GAL4 DNA binding domain. The amino acid and nucleic acid sequences for Gal4 may be found at Genbank Accession No. K01486.

20 In one embodiment of the fusion proteins described herein, the transcription activation domain is a GAL4 transcription activation domain. In one embodiment, the peptide having a transcription activation domain comprises GAL4 amino acid residues 768-881. The portion of Gal4 which corresponds to
25 amino acid residues 768-881 may comprise a Gal4 activation domain.

In one embodiment of the fusion proteins described herein, the transcription activation domain is a VP16 transcription
30 activation domain. The amino acid and nucleic acid sequences

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throughout the specification to indicate specific nucleotides: C=cytosine; A=adenosine; T=thymidine; G=guanosine; and U=uracil.

- 5 In one embodiment of the fusion proteins described herein, the fusion protein comprises an influenza hemagglutinin (HA) epitope tag. The sequence for influenza hemagglutinin (HA) epitope may be found in Genbank Accession No. U29899 at nucleotide bases 5042-5068 within the plasmid pACT2. The
10 invention may also comprise other types of epitope tags known to one skilled in the art.

In one embodiment of the fusion proteins described herein, the reporter gene is a LacZ reporter gene. The amino acid and
15 nucleic acid sequences for LacZ may be found at Genbank Accession no. U89671.

- In one embodiment of the methods described herein, (a) the fusion protein expressed by the first plasmid comprises a
20 peptide comprising a LexA protein DNA binding domain, wherein the p66 subunit polypeptide is bound at its C-terminal amino acid to the N-terminal amino acid of the peptide comprising a LexA protein DNA binding domain; and (b) the fusion protein expressed by the second plasmid comprises a Gal4 peptide
25 corresponding to amino acids 768-881 of Gal4, and an influenza hemagglutinin (HA) epitope tag, which Gal4 peptide is bound at its C-terminal amino acid to the N-terminal amino acid of the influenza hemagglutinin (HA) epitope tag, which influenza hemagglutinin (HA) epitope tag is bound at its C-
30 terminal amino acid to the N-terminal amino acid of the p51

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amino acids 768-881 of Gal4, and an influenza hemagglutinin (HA) epitope tag, which Gal4 peptide is bound at its C-terminal amino acid to the N-terminal amino acid of the influenza hemagglutinin (HA) epitope tag, which influenza
5 hemagglutinin (HA) epitope tag is bound at its C-terminal amino acid to the N-terminal amino acid of the p51 subunit polypeptide.

In one embodiment of the methods described herein, (a) the
10 fusion protein expressed by the first plasmid comprises a LexA peptide corresponding to amino acid residues 1-87, wherein the LexA peptide is bound at its C-terminal amino acid to the N-terminal amino acid of the of the p66 subunit polypeptide; and (b) the fusion protein expressed by the
15 second plasmid comprises a Gal4 peptide corresponding to amino acids 768-881 of Gal4, which Gal4 peptide is bound at its C-terminal amino acid to the N-terminal amino acid of the p51 subunit polypeptide.

20 In one embodiment of the methods described herein, (a) the fusion protein expressed by the first plasmid comprises a LexA peptide corresponding to amino acid residues 1-202, and a peptide comprising six consecutive alanine residues, wherein the LexA peptide is bound at its C-terminal amino
25 acid to the N-terminal amino acid of the peptide comprising six consecutive alanine residues, wherein the peptide comprising six consecutive alanine residues is bound at its C-terminal amino acid to the N-terminal amino acid of the p66 subunit polypeptide; and (b) the fusion protein expressed by
30 the second plasmid comprises a Gal4 peptide corresponding to

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epitope tag, wherein the influenza hemagglutinin (HA) epitope tag is bound at its C-terminal amino acid to the N-terminal amino acid of the peptide comprising six consecutive alanine residues, wherein the peptide comprising six consecutive
5 alanine residues is bound at its C-terminal amino acid to the N-terminal amino acid of the p66 subunit polypeptide; and (b) the fusion protein expressed by second plasmid comprises a peptide comprising a LexA protein DNA binding domain, wherein the p51 subunit polypeptide is bound at its C-terminal amino
10 acid to the N-terminal amino acid of the peptide comprising a LexA protein DNA binding domain.

In one embodiment of the methods described herein, (a) the fusion protein expressed by the first plasmid comprises a
15 Gal4 peptide corresponding to amino acids 768-881 of Gal4, an influenza hemagglutinin (HA) epitope tag, and a peptide comprising six consecutive alanine residues, wherein the Gal4 peptide is bound at its C-terminal amino acid to the N-terminal amino acid of the influenza hemagglutinin (HA)
20 epitope tag, wherein the influenza hemagglutinin (HA) epitope tag is bound at its C-terminal amino acid to the N-terminal amino acid of the peptide comprising six consecutive alanine residues, wherein the peptide comprising six consecutive alanine residues is bound at its C-terminal amino acid to the
25 N-terminal amino acid of the p66 subunit polypeptide; and (b) the fusion protein expressed by second plasmid comprises a peptide comprising a LexA protein DNA binding domain, wherein peptide comprising a LexA protein DNA binding domain is bound at its C-terminal amino acid to the N-terminal amino acid of
30 the p51 subunit polypeptide.

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In one embodiment of the methods described herein, (a) the fusion protein expressed by the first plasmid comprises a Gal4 peptide corresponding to amino acids 768-881 of Gal4, an influenza hemagglutinin (HA) epitope tag, and a peptide
 5 comprising six consecutive alanine residues, wherein the Gal4 peptide is bound at its C-terminal amino acid to the N-terminal amino acid of the influenza hemagglutinin (HA) epitope tag, wherein the influenza hemagglutinin (HA) epitope tag is bound at its C-terminal amino acid to the N-terminal
 10 amino acid of the peptide comprising six consecutive alanine residues, wherein the peptide comprising six consecutive alanine residues is bound at its C-terminal amino acid to the N-terminal amino acid of the p66 subunit polypeptide; and (b) the fusion protein expressed by second plasmid comprises a
 15 peptide comprising a Gal4 protein DNA binding domain, which peptide comprising a Gal4 protein DNA binding domain is bound at its C-terminal amino acid to the N-terminal amino acid of the p51 subunit polypeptide.

20 In one embodiment of the methods described herein, (a) the fusion protein expressed by the first plasmid comprises a Gal4 peptide corresponding to amino acids 768-881 of Gal4, wherein the Gal4 peptide is bound at its C-terminal amino acid to the N-terminal amino acid of the p66 subunit
 25 polypeptide; and (b) the fusion protein expressed by second plasmid comprises a peptide comprising a LexA protein DNA binding domain, wherein the p51 subunit polypeptide is bound at its C-terminal amino acid to the N-terminal amino acid of the peptide comprising a LexA protein DNA binding domain.

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hemagglutinin (HA) epitope tag, and a peptide comprising six consecutive alanine residues, wherein the Gal4 peptide is bound at its C-terminal amino acid to the N-terminal amino acid of the influenza hemagglutinin (HA) epitope tag, wherein
5 the influenza hemagglutinin (HA) epitope tag is bound at its C-terminal amino acid to the N-terminal amino acid of the peptide comprising six consecutive alanine residues, wherein the peptide comprising six consecutive alanine residues is bound at its C-terminal amino acid to the N-terminal amino
10 acid of the p66 subunit polypeptide.

This invention provides a method of making a pharmaceutical composition which comprises:

- a) determining whether a compound inhibits HIV-1 reverse
15 transcriptase by one of the methods described herein;
- b) recovering the compound if it is determined to inhibit HIV-1 reverse transcriptase; and
- c) admixing the compound with a pharmaceutically acceptable carrier.

20

As used herein, "inhibits" means that the amount is reduced as compared with the amount that would occur in a control sample without the compound. As used herein "enhanced" means that the amount is increased compared with the amount that would occur
25 in a control sample without the compound.

As used herein, the term "compound" includes both protein and non-protein moieties. In one embodiment, the compound is a small molecule. In another embodiment, the compound is a
30 protein. The protein may be, by way of example, an antibody

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directed against a portion of a p51 or p66 subunit. The agent may be derived from a library of low molecular weight compounds or a library of extracts from plants or other organisms. In an embodiment, the agent is known. In a
 5 separate embodiment, the agent is not previously known. The agents of the subject invention include but are not limited to compounds or molecular entities such as peptides, polypeptides, and other organic or inorganic molecules and combinations thereof

10

In one embodiment of the methods described herein, the compound is an antibody or a portion of an antibody. In one embodiment of the antibody, the antibody is a monoclonal antibody. In one embodiment of the antibody, the antibody is
 15 a polyclonal antibody. In one embodiment of the antibody, the antibody is a humanized antibody. In one embodiment of the antibody, the antibody is a chimeric antibody. The portion of the antibody may comprise a light chain of the antibody. The portion of the antibody may comprise a heavy chain of the
 20 antibody. The portion of the antibody may comprise a Fab portion of the antibody. The portion of the antibody may comprise a $F(ab')_2$ portion of the antibody. The portion of the antibody may comprise a Fd portion of the antibody. The portion of the antibody may comprise a Fv portion of the
 25 antibody. The portion of the antibody may comprise a variable domain of the antibody. The portion of the antibody may comprise one or more CDR domains of the antibody.

In one embodiment of the methods described herein, the
 30 compound is a polypeptide. In one embodiment of the methods

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described herein, the compound is a oligopeptide. In one
embodiment of the methods described herein, the compound is
a nonpeptidyl agent. In one embodiment, the nonpeptidyl agent
is a compound having a molecular weight less than 500
5 daltons.

In one embodiment of the methods described herein, the
reverse HIV-1 transcriptase enzyme or its p51 and p66
subunits is present in a subject and the contacting is
10 effected by administering the compound to the subject.
Accordingly, the subject invention has various applications
which includes HIV treatment such as treating a subject who
has become afflicted with HIV. As used herein, "afflicted
with HIV" means that the subject has at least one cell which
15 has been infected by HIV. As used herein, "treating" means
either slowing, stopping or reversing the progression of an
HIV disorder. In the preferred embodiment, "treating" means
reversing the progression to the point of eliminating the
disorder. As used herein, "treating" also means the reduction
20 of the number of viral infections, reduction of the number of
infectious viral particles, reduction of the number of
virally infected cells, or the amelioration of symptoms
associated with HIV. Another application of the subject
invention is to prevent a subject from contracting HIV. As
25 used herein, "contracting HIV" means becoming infected with
HIV, whose genetic information replicates in and/or
incorporates into the host cells. Another application of the
subject invention is to treat a subject who has become
infected with HIV. As used herein, "HIV infection" means the
30 introduction of HIV genetic information into a target cell,

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The dose of the agent or composition of the invention will vary depending on the subject and upon the particular route of administration used. Dosages can range from 0.1 to 100,000 $\mu\text{g/kg}$. Based upon the composition, the dose can be delivered continuously, such as by continuous pump, or at periodic intervals. For example, on one or more separate occasions. Desired time intervals of multiple doses of a particular composition can be determined without undue experimentation by one skilled in the art.

10

As used herein, "effective dose" means an amount in sufficient quantities to either treat the subject or prevent the subject from becoming HIV infected. A person of ordinary skill in the art can perform simple titration experiments to determine what amount is required to treat the subject. As used herein, "contracting HIV" means becoming infected with HIV, whose genetic information replicates in and/or incorporates into the host cells. In one embodiment, the effective amount of the agent or composition comprises from about 0.000001 mg/kg body weight to about 100 mg/kg body weight of the subject.

20

As used herein, "subject" means any animal or artificially modified animal capable of becoming HIV-infected. The subjects include but are not limited to a human being, a primate, an equine, an opine, an avian, a bovine, a porcine, a canine, a feline or a mouse. Artificially modified animals include, but are not limited to, SCID mice with human immune systems. The animals include but are not limited to mice, rats, dogs, guinea pigs, ferrets, rabbits, and primates. In the preferred embodiment, the subject is a human being. The

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administered daily. In one embodiment of the methods described herein, the agent is administered every other day. In one embodiment of the methods described herein, the agent is administered every 6 to 8 days. In one embodiment of the methods described herein, the agent is administered weekly.

This invention provides a method of inhibiting formation of a complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase, which comprises contacting either (1) the p51 subunit polypeptide, (2) the p66 subunit polypeptide, or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of a compound determined to do so by the method of claim 2, so to thereby inhibit formation of a complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase.

This invention provides a method of enhancing formation of a complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase, which comprises contacting either (1) the p51 subunit polypeptide, (2) the p66 subunit polypeptide, or (3) both the p51 subunit polypeptide and the p66 subunit polypeptide, with an effective amount of a compound determined to do so by the method of claim 4, so to thereby enhance formation of a complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and a p66 subunit polypeptide of HIV-1 reverse transcriptase.

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skilled in the art including but not limited to those wherein
the compound is administered orally, intravenously,
subcutaneously, intramuscularly, topically or by liposome-
mediated delivery. The subject may be any subject including
5 but not limited to a human being, a primate, an equine, an
opine, an avian, a bovine, a porcine, a canine, a feline or
a mouse. In one embodiment, the compound is administered at
least once per day. In one embodiment, the compound is
administered daily. In one embodiment, the compound is
10 administered every other day. In one embodiment, compound is
administered every 6 to 8 days. In one embodiment, the
compound is administered weekly.

This invention provides a compound determined to be capable
15 of inhibiting formation of a complex between a p51 subunit
polypeptide of HIV-1 reverse transcriptase and a p66 subunit
polypeptide of HIV-1 reverse transcriptase.

This invention provides a compound determined to be capable
20 of enhancing formation of a complex between a p51 subunit
polypeptide of HIV-1 reverse transcriptase and a p66 subunit
polypeptide of HIV-1 reverse transcriptase.

This invention provides a compound determined to be capable
25 of inhibiting formation of a complex between a first p66
subunit polypeptide of HIV-1 reverse transcriptase and a
second p66 subunit polypeptide of HIV-1 reverse
transcriptase.

30 This invention provides a compound determined to be capable

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of enhancing formation of a complex between a first p66 subunit polypeptide of HIV-1 reverse transcriptase and a second p66 subunit polypeptide of HIV-1 reverse transcriptase.

5

This invention provides a composition which comprises one of the compounds described herein and a carrier. As used herein, "composition" means a mixture. The compositions include but are not limited to those suitable for oral, rectal, 10 intravaginal, topical, nasal, ophthalmic, or parenteral, intravenous, subcutaneous, intramuscular, and intraperitoneal administration to a subject. As used herein, "parenteral" includes but is not limited to subcutaneous, intravenous, intramuscular, or intrasternal injections or infusion 15 techniques.

This invention provides an agent or composition described herein and a carrier. Such carrier may be one that is a pharmaceutically acceptable carrier. Pharmaceutically 20 acceptable carriers are well known to those skilled in the art. Such pharmaceutically acceptable carriers may include but are not limited to aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils 25 such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium 30 chloride, lactated Ringer's or fixed oils. Intravenous

vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, 5 chelating agents, inert gases and the like.

In one embodiment of the agents described herein, the compound is an antibody or portion of an antibody. As used herein, "antibody" means an immunoglobulin molecule comprising 10 two heavy chains and two light chains and which recognizes an antigen. The immunoglobulin molecule may derive from any of the commonly known classes, including but not limited to IgA, secretory IgA, IgG and IgM. IgG subclasses are also well known to those in the art and include but are not limited to 15 human IgG1, IgG2, IgG3 and IgG4. It includes, by way of example, both naturally occurring and non-naturally occurring antibodies. Specifically, "antibody" includes polyclonal and monoclonal antibodies, and monovalent and divalent fragments thereof. Furthermore, "antibody" includes chimeric antibodies, 20 wholly synthetic antibodies, single chain antibodies, and fragments thereof. Optionally, an antibody can be labeled with a detectable marker. Detectable markers include, for example, radioactive or fluorescent markers. The antibody may be a human or nonhuman antibody. The nonhuman antibody may be 25 humanized by recombinant methods to reduce its immunogenicity in man. Methods for humanizing antibodies are known to those skilled in the art. As used herein, "monoclonal antibody," also designated as mAb, is used to describe antibody molecules whose primary sequences are essentially identical 30 and which exhibit the same antigenic specificity. Monoclonal

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antibodies may be produced by hybridoma, recombinant, transgenic or other techniques known to one skilled in the art. The term "antibody" includes, but is not limited to, both naturally occurring and non-naturally occurring
 5 antibodies. Specifically, the term "antibody" includes polyclonal and monoclonal antibodies, and antigen-binding fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies, wholly synthetic antibodies, and antigen-binding fragments thereof. Accordingly, in one
 10 embodiment, the antibody is a monoclonal antibody. In one embodiment, the antibody is a polyclonal antibody. In one embodiment, the antibody is a humanized antibody. In one embodiment, the antibody is a chimeric antibody. Such chimeric antibodies may comprise a portion of an antibody
 15 from one source and a portion of an antibody from another source.

In one embodiment, the portion of the antibody comprises a light chain of the antibody. As used herein, "light chain"
 20 means the smaller polypeptide of an antibody molecule composed of one variable domain (VL) and one constant domain (CL), or fragments thereof. In one embodiment, the portion of the antibody comprises a heavy chain of the antibody. As used herein, "heavy chain" means the larger polypeptide of an
 25 antibody molecule composed of one variable domain (VH) and three or four constant domains (CH1, CH2, CH3, and CH4), or fragments thereof. In one embodiment, the portion of the antibody comprises a Fab portion of the antibody. As used herein, "Fab" means a monovalent antigen binding fragment of
 30 an immunoglobulin that consists of one light chain and part

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of a heavy chain. It can be obtained by brief papain digestion or by recombinant methods. In one embodiment, the portion of the antibody comprises a $F(ab')_2$ portion of the antibody. As used herein, " $F(ab')_2$ fragment" means a bivalent
5 antigen binding fragment of an immunoglobulin that consists of both light chains and part of both heavy chains. It can be obtained by brief pepsin digestion or recombinant methods. In one embodiment, the portion of the antibody comprises a F_d portion of the antibody. In one embodiment, the portion of
10 the antibody comprises a F_v portion of the antibody. In one embodiment, the portion of the antibody comprises a variable domain of the antibody. In one embodiment, the portion of the antibody comprises a constant domain of the antibody. In one embodiment, the portion of the antibody comprises one or more
15 CDR domains of the antibody. As used herein, "CDR" or "complementarity determining region" means a highly variable sequence of amino acids in the variable domain of an antibody.

20 This invention provides humanized forms of the antibodies described herein. As used herein, "humanized" describes antibodies wherein some, most or all of the amino acids outside the CDR regions are replaced with corresponding amino acids derived from human immunoglobulin molecules. In one
25 embodiment of the humanized forms of the antibodies, some, most or all of the amino acids outside the CDR regions have been replaced with amino acids from human immunoglobulin molecules but where some, most or all amino acids within one or more CDR regions are unchanged. Small additions,
30 deletions, insertions, substitutions or modifications of

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immunoglobulins. These have one or more CDRs and possible additional amino acids from a donor immunoglobulin and a framework region from an accepting human immunoglobulin. These patents describe a method to increase the affinity of an antibody for the desired antigen. Some amino acids in the framework are chosen to be the same as the amino acids at those positions in the donor rather than in the acceptor. Specifically, these patents describe the preparation of a humanized antibody that binds to a receptor by combining the CDRs of a mouse monoclonal antibody with human immunoglobulin framework and constant regions. Human framework regions can be chosen to maximize homology with the mouse sequence. A computer model can be used to identify amino acids in the framework region which are likely to interact with the CDRs or the specific antigen and then mouse amino acids can be used at these positions to create the humanized antibody.

The above patents 5,585,089 and 5,693,761, and WO 90/07861 also propose four possible criteria which may be used in designing the humanized antibodies. The first proposal was that for an acceptor, use a framework from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin to be humanized, or use a consensus framework from many human antibodies. The second proposal was that if an amino acid in the framework of the human immunoglobulin is unusual and the donor amino acid at that position is typical for human sequences, then the donor amino acid rather than the acceptor may be selected. The third proposal was that in the positions immediately adjacent to the 3 CDRs in the humanized immunoglobulin chain, the donor amino acid rather

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than the acceptor amino acid may be selected. The fourth proposal was to use the donor amino acid residue at the framework positions at which the amino acid is predicted to have a side chain atom within 3Å of the CDRs in a three dimensional model of the antibody and is predicted to be
5 capable of interacting with the CDRs. The above methods are merely illustrative of some of the methods that one skilled in the art could employ to make humanized antibodies.

10 This invention provides isolated nucleic acids encoding the antibodies described herein or their humanized versions. The nucleic acid can be RNA, DNA or cDNA. In one embodiment, the nucleic acid encodes the light chain. In one embodiment, the nucleic acid encodes the heavy chain. In one embodiment, the
15 nucleic acid encodes both the heavy and light chains. In one embodiment, one or more nucleic acids encode the Fab portion. In one embodiment, one or more nucleic acids encode CDR portions. In one embodiment, the nucleic acid encodes the variable domain.

20

In one embodiment of the agents described herein, the agent is a polypeptide. In one embodiment of the agents described herein, the agent is a oligopeptide. As used herein, "polypeptide" means two or more amino acids linked by a
25 peptide bond.

The nucleic acids, polypeptides and antibodies described herein may be isolated and/or purified. One skilled in the art would know how to isolate and/or purify them. Methods are
30 provided in any laboratory manual such as "Molecular Cloning"

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transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase which comprises: a) contacting a yeast cell cotransformed with a first plasmid which expresses in the cell a fusion protein comprising the p66 subunit polypeptide of HIV-1 reverse transcriptase and a second plasmid which expresses in the cell a fusion protein comprising the p51 subunit polypeptide of HIV-1 reverse transcriptase with the compound wherein the cell further comprises a reporter gene which is activated in the presence of a complex between the p66 subunit polypeptide and the p51 subunit polypeptide; b) determining the level of activity of the reporter gene in the cell in the presence of the compound; and c) comparing the level of activity of the reporter gene determined in step (b) with the level of activity of the reporter gene in the absence of the compound, wherein a decreased level of activity of the reporter gene indicates that the compound is an inhibitor of the formation of the complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and the p66 subunit polypeptide of HIV-1 reverse transcriptase.

20

In an embodiment of the above-described method of testing a compound to determine whether it is an inhibitor of formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises full length bacterial protein LexA fused to the p66 subunit polypeptide of HIV-1 reverse transcriptase at amino acid position 1 of the N-terminal amino acid sequence of the full length bacterial protein LexA and the fusion protein expressed by the second plasmid

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5 epitope tag, wherein the p51 subunit polypeptide is fused to
the second end of the influenza HA epitope tag.

10 formation of a complex between a p66 subunit polypeptide of
HIV-1 reverse transcriptase and a p51 subunit polypeptide of
HIV-1 reverse transcriptase, the fusion protein expressed by
the first plasmid further comprises full length bacterial
protein LexA fused to the p66 subunit polypeptide of HIV-1
15 reverse transcriptase at amino acid position 1 of the N-
terminal amino acid sequence of the full length bacterial
protein LexA and the fusion protein expressed by the second
plasmid further comprises amino acids at positions 768-881 of
the C-terminal amino acid sequence of Gal4AD fused at
20 position 881 to the p51 subunit polypeptide of HIV-1 reverse
transcriptase.

formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises amino acids 1-87 of the LexA DNA binding domain fused to the p66 subunit polypeptide of HIV-1 reverse transcriptase at amino acid position 87 and

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the fusion protein expressed by the second plasmid further comprises amino acids at positions 768-881 of the C-terminal amino acid sequence of Gal4AD, wherein the C-terminal amino acid sequence of Gal4AD is fused at amino acid position 881
5 to one end of an influenza hemagglutinin (HA) epitope tag, wherein the p51 subunit polypeptide is fused to the second end of the influenza HA epitope tag.

In a further embodiment of the above-described method of
10 testing a compound to determine whether it is an inhibitor of formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises amino acids 1-87 of the
15 LexA DNA binding domain fused to the p66 subunit polypeptide of HIV-1 reverse transcriptase at amino acid position 87 and the fusion protein expressed by the second plasmid further comprises amino acids at positions 768-881 of the C-terminal amino acid sequence of Gal4AD fused at position 881 to the
20 p51 subunit polypeptide of HIV-1 reverse transcriptase.

In a still further embodiment of the above-described method of testing a compound to determine whether it is an inhibitor of formation of a complex between a p66 subunit polypeptide
25 of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises full length bacterial protein LexA protein fused at amino acid position 202 to a first end of a six alanine linker, wherein the p66 subunit
30 polypeptide is fused at amino acid 1 to the second end of the

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six alanine linker and the fusion protein expressed by the second plasmid expresses further comprises amino acids at positions 768-881 of the C-terminal amino acid sequence of Gal4AD fused at position 881 to the p51 subunit polypeptide
5 of HIV-1 reverse transcriptase.

In yet another embodiment of the above-described method of testing a compound to determine whether it is an inhibitor of formation of a complex between a p66 subunit polypeptide of
10 HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises full length bacterial protein LexA protein fused at amino acid position 202 to one end of a six alanine linker and the p66 subunit polypeptide
15 fused at amino acid 1 to the other end of the six alanine linker and the fusion protein expressed by the second plasmid further comprises amino acids at positions 768-881 of the C-terminal amino acid sequence of Gal4AD wherein said C-terminal sequence is fused at amino acid position 881 to one
20 end of an influenza hemagglutinin (HA) epitope tag and the p51 subunit polypeptide is fused at the other end of the HA epitope tag.

In another embodiment of the above-described method of
25 testing a compound to determine whether it is an inhibitor of formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises amino acids at positions
30 768-881 of the C-terminal amino acid sequence of Gal4AD fused

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to a first end of an influenza hemagglutinin (HA) epitope and a six alanine linker fused at a first end to the second end of the influenza hemagglutinin (HA) epitope, wherein the p66 subunit polypeptide of HIV-1 reverse transcriptase is fused at amino acid 1 to the second end of the six alanine linker and the fusion protein expressed by second plasmid further comprises full length LexA bacterial protein LexA fused at amino acid 1 to the p51 subunit polypeptide of HIV-1 reverse transcriptase.

10

In a further embodiment of the above-described method of testing a compound to determine whether it is an inhibitor of formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises amino acids at positions 768-881 of the C-terminal amino acid sequence of Gal4AD fused to a first end of an influenza hemagglutinin (HA) epitope and a six alanine linker fused at a first end to the second end of the influenza hemagglutinin (HA) epitope, wherein the p66 subunit polypeptide of HIV-1 reverse transcriptase is fused at amino acid 1 to the second end of the six alanine linker and the fusion protein expressed by the second plasmid further comprises the LexA DNA binding domain fused to amino acid position 1 of the p51 subunit polypeptide of HIV-1 reverse transcriptase.

In a further embodiment of the above-described method of testing a compound to determine whether it is an inhibitor of formation of a complex between a p66 subunit polypeptide of

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HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises amino acids at positions 768-881 of the C-terminal amino acid sequence of Gal4AD fused
 5 to a first end of an influenza hemagglutinin (HA) epitope and a six alanine linker fused at a first end to the second end of the influenza hemagglutinin (HA) epitope, wherein the p66 subunit polypeptide of HIV-1 reverse transcriptase is fused at amino acid 1 to the second end of the six alanine linker
 10 and the fusion protein expressed by the second plasmid further comprises the GAL4 DNA binding domain fused to amino acid position 1 of the p51 subunit polypeptide of HIV-1 reverse transcriptase.

15 In still another embodiment of the above-described method of testing a compound to determine whether it is an inhibitor of formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by
 20 the first plasmid further comprises amino acids at positions 768-881 of the C-terminal amino acid sequence of Gal4AD, wherein the C-terminal amino acid sequence of Gal4AD is fused at amino acid position 881 to the p66 subunit polypeptide of HIV-1 reverse transcriptase, and the fusion protein expressed
 25 by second plasmid further comprises full length LexA bacterial protein LexA fused at amino acid position 1 to the p51 subunit polypeptide of HIV-1 reverse transcriptase.

In another embodiment of the above-described method of
 30 testing a compound to determine whether it is an inhibitor of

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formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises amino acids at positions
5 768-881 of the C-terminal amino acid sequence of Gal4AD fused at amino acid position 881 to the p66 subunit polypeptide of HIV-1 reverse transcriptase and the fusion protein expressed by the second plasmid further comprises the LexA DNA binding domain fused to amino acid position 1 of the p51 subunit
10 polypeptide of HIV-1 reverse transcriptase.

In a further embodiment of the above-described method of testing a compound to determine whether it is an inhibitor of formation of a complex between a p66 subunit polypeptide of
15 HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises amino acids at positions 768-881 of the C-terminal amino acid sequence of Gal4AD fused at amino acid position 881 to the p66 subunit polypeptide of
20 HIV-1 reverse transcriptase and the fusion protein expressed by the second plasmid further comprises the GAL4 DNA binding domain fused to amino acid position 1 of the p51 subunit polypeptide of HIV-1 reverse transcriptase.

25 One of skill will readily be able to make or use the plasmids described herein using the known nucleic acid sequence for HIV-1 reverse transcriptase, and the p66 subunit polypeptide thereof or the p51 subunit polypeptide thereof which may be comprised in expression vectors made by one of ordinary skill
30 in the art; and make or purchase the vectors used herein

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reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase which comprises: a) contacting a yeast cell cotransformed with a first plasmid which expresses in the cell a fusion protein comprising the p66 subunit polypeptide of HIV-1 reverse transcriptase and a second plasmid which expresses in the cell a fusion protein comprising the p51 subunit polypeptide of HIV-1 reverse transcriptase with the compound wherein the cell further comprises a reporter gene which is activated in the presence of a complex between the p66 subunit polypeptide and the p51 subunit polypeptide; b) determining the level of activity of the reporter gene in the cell in the presence of the compound; and c) comparing the level of activity of the reporter gene determined in step (b) with the level of activity of the reporter gene in the absence of the compound, wherein an increased level of activity of the reporter gene indicates that the compound is an activator of the formation of the complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and the p66 subunit polypeptide of HIV-1 reverse transcriptase.

In an embodiment of the above-described method of testing a compound to determine whether it is an activator of formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises full length bacterial protein LexA fused to the p66 subunit polypeptide of HIV-1 reverse transcriptase at amino acid position 1 of the N-terminal amino acid sequence of the full length bacterial protein LexA

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and the fusion protein expressed by the second plasmid further comprises amino acids at positions 768-881 of the C-terminal amino acid sequence of Gal4AD, wherein the C-terminal amino acid sequence of Gal4AD is fused at amino acid
5 position 881 to one end of an influenza hemagglutinin (HA) epitope tag, wherein the p51 subunit polypeptide is fused to the second end of the influenza HA epitope tag.

In another embodiment of the above-described method of
10 testing a compound to determine whether it is an activator of formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises full length bacterial
15 protein LexA fused to the p66 subunit polypeptide of HIV-1 reverse transcriptase at amino acid position 1 of the N-terminal amino acid sequence of the full length bacterial protein LexA and the fusion protein expressed by the second plasmid further comprises amino acids at positions 768-881 of
20 the C-terminal amino acid sequence of Gal4AD fused at position 881 to the p51 subunit polypeptide of HIV-1 reverse transcriptase.

In a further embodiment of the above-described method of
25 testing a compound to determine whether it is an activator of formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises amino acids 1-87 of the
30 LexA DNA binding domain fused to the p66 subunit polypeptide

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polypeptide is fused at amino acid 1 to the second end of the six alanine linker and the fusion protein expressed by the second plasmid expresses further comprises amino acids at positions 768-881 of the C-terminal amino acid sequence of Gal4AD fused at position 881 to the p51 subunit polypeptide of HIV-1 reverse transcriptase.

In another embodiment of the above-described method of testing a compound to determine whether it is an activator of formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises full length bacterial protein LexA protein fused at amino acid position 202 to one end of a six alanine linker and the p66 subunit polypeptide fused at amino acid 1 to the other end of the six alanine linker and the fusion protein expressed by the second plasmid further comprises amino acids at positions 768-881 of the C-terminal amino acid sequence of Gal4AD wherein said C-terminal sequence is fused at amino acid position 881 to one end of an influenza hemagglutinin (HA) epitope tag and the p51 subunit polypeptide is fused at the other end of the HA epitope tag.

In a further embodiment of the above-described method of testing a compound to determine whether it is an activator of formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises amino acids at positions

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768-881 of the C-terminal amino acid sequence of Gal4AD fused to a first end of an influenza hemagglutinin (HA) epitope and a six alanine linker fused at a first end to the second end of the influenza hemagglutinin (HA) epitope, wherein the p66
5 subunit polypeptide of HIV-1 reverse transcriptase is fused at amino acid 1 to the second end of the six alanine linker and the fusion protein expressed by second plasmid further comprises full length LexA bacterial protein LexA fused at amino acid 1 to the p51 subunit polypeptide of HIV-1 reverse
10 transcriptase.

In another embodiment of the above-described method of testing a compound to determine whether it is an activator of formation of a complex between a p66 subunit polypeptide of
15 HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises amino acids at positions 768-881 of the C-terminal amino acid sequence of Gal4AD fused to a first end of an influenza hemagglutinin (HA) epitope and
20 a six alanine linker fused at a first end to the second end of the influenza hemagglutinin (HA) epitope, wherein the p66 subunit polypeptide of HIV-1 reverse transcriptase is fused at amino acid 1 to the second end of the six alanine linker and the fusion protein expressed by the second plasmid
25 further comprises the LexA DNA binding domain fused to amino acid position 1 of the p51 subunit polypeptide of HIV-1 reverse transcriptase.

In still another embodiment of the above-described method of
30 testing a compound to determine whether it is an activator of

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testing a compound to determine whether it is an activator of formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by
5 the first plasmid further comprises amino acids at positions 768-881 of the C-terminal amino acid sequence of Gal4AD fused at amino acid position 881 to the p66 subunit polypeptide of HIV-1 reverse transcriptase and the fusion protein expressed by the second plasmid further comprises the LexA DNA binding
10 domain fused to amino acid position 1 of the p51 subunit polypeptide of HIV-1 reverse transcriptase.

In a further embodiment of the above-described method of testing a compound to determine whether it is an activator of
15 formation of a complex between a p66 subunit polypeptide of HIV-1 reverse transcriptase and a p51 subunit polypeptide of HIV-1 reverse transcriptase, the fusion protein expressed by the first plasmid further comprises amino acids at positions 768-881 of the C-terminal amino acid sequence of Gal4AD fused
20 at amino acid position 881 to the p66 subunit polypeptide of HIV-1 reverse transcriptase and the fusion protein expressed by the second plasmid further comprises the GAL4 DNA binding domain fused to amino acid position 1 of the p51 subunit polypeptide of HIV-1 reverse transcriptase.

25

The activators determined by the above-described methods are useful for the preparation of drugs, as pharmaceutical compositions, which will enhance complex formation prematurely or inappropriately between the p66 subunit
30 polypeptide of HIV-1 reverse transcriptase and the p51

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subunit polypeptide of HIV-1 reverse transcriptase so as to
kill the HIV-1 virus or render the the HIV-1 virus inactive
or incapable of infecting cells of a subject, i.e. lack the
functions of an infected HIV-1 virus, including human
5 subjects.

This invention also provides a method of making a
pharmaceutical composition comprising an activator of the
formation of the complex between the p66 subunit polypeptide
10 of HIV-1 reverse transcriptase and the p51 subunit
polypeptide of HIV-1 reverse transcriptase which comprises:
a) determining whether a compound is an activator of the
formation of the complex between the p66 subunit polypeptide
of HIV-1 reverse transcriptase and the p51 subunit
15 polypeptide of HIV-1 reverse transcriptase according to a
method which comprises: i) contacting a yeast cell
cotransformed with a first plasmid which expresses in the
cell a fusion protein comprising the p66 subunit polypeptide
of HIV-1 reverse transcriptase and a second plasmid which
20 expresses in the cell a fusion protein comprising the p51
subunit polypeptide of HIV-1 reverse transcriptase with the
compound wherein the cell further comprises a reporter gene
which is activated in the presence of a complex between the
p66 subunit polypeptide and the p51 subunit polypeptide; ii)
25 determining the level of activity of the reporter gene in the
cell in the presence of the compound; and iii) comparing the
level of activity of the reporter gene determined in step
(ii) with the level of activity of the reporter gene in the
absence of the compound, wherein an increased level of
30 activity of the reporter gene indicates that the compound is

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an activator of the formation of the complex between the p51 subunit polypeptide of HIV-1 reverse transcriptase and the p66 subunit polypeptide of HIV-1 reverse transcriptase; and b) admixing the compound determined to be the activator in step (a)(iii) with a pharmaceutically acceptable carrier. Any of the above-described methods to determine whether a compound is an activator of the formation of the complex between the p66 subunit polypeptide of HIV-1 reverse transcriptase and the p51 subunit polypeptide of HIV-1 reverse transcriptase may be used in the method of making a pharmaceutical composition comprising the determined activator compound, but is not limited thereto, since one of skill will readily be able to substitute well known reporter genes for the reporter genes used in the examples herein. Moreover, one of skill is not limited to using the yeast cells exemplified in any of the above-described methods herein, but may modify the methods to use other eukaryotic cells, mammalian cells or cell lines such as 298 T cells.

This invention further provides a method of testing a compound to determine whether it is an inhibitor of formation of a complex between a first p66 subunit polypeptide of HIV-1 reverse transcriptase and a second p66 subunit polypeptide of HIV-1 reverse transcriptase which comprises: a) contacting a yeast cell cotransformed with a first plasmid which expresses in the cell a fusion protein comprising the first p66 subunit polypeptide of HIV-1 reverse transcriptase and a second plasmid which expresses in the cell a fusion protein comprising the second p66 subunit polypeptide of HIV-1 reverse transcriptase with the compound wherein the cell

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at the second end of the six alanine linker.

This invention also provides a method of making a pharmaceutical composition comprising an inhibitor of
5 formation of a complex between a first p66 subunit polypeptide of HIV-1 reverse transcriptase and a second p66 subunit polypeptide of HIV-1 reverse transcriptase which comprises: a) determining whether a compound is an inhibitor
10 of formation of a complex between a first p66 subunit polypeptide of HIV-1 reverse transcriptase and a second p66 subunit polypeptide of HIV-1 reverse transcriptase according to a method which comprises: i) contacting a yeast cell cotransformed with a first plasmid which expresses in the cell a fusion protein comprising the first p66 subunit
15 polypeptide of HIV-1 reverse transcriptase and a second plasmid which expresses in the cell a fusion protein comprising the second p66 subunit polypeptide of HIV-1 reverse transcriptase with the compound wherein the cell further comprises a reporter gene which is activated in the
20 presence of a complex between the first p66 subunit polypeptide and the second p66 subunit polypeptide; ii) determining the level of activity of the reporter gene in the cell in the presence of the compound; and iii) comparing the level of activity of the reporter gene determined in step
25 (ii) with the level of activity of the reporter gene in the absence of the compound, wherein a decreased level of activity of the reporter gene indicates that the compound is an inhibitor of the formation of the complex between the first p66 subunit polypeptide of HIV-1 reverse transcriptase
30 and the second p66 subunit polypeptide of HIV-1 reverse

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N-terminal amino acid sequence of the full length bacterial protein LexA and the fusion protein expressed by second plasmid further comprises amino acids at positions 768-881 of the C-terminal amino acid sequence of Gal4AD fused to one end
 5 of an influenza hemagglutinin (HA) epitope and a six alanine linker fused at a first end to the second end of the influenza hemagglutinin (HA) epitope, wherein the second p66 subunit polypeptide of HIV-1 reverse transcriptase is fused at the second end of the six alanine linker.

10

The activators determined by the above-described methods are useful for the preparation of drugs, as pharmaceutical compositions, which will enhance complex formation prematurely or inappropriately between the first p66 subunit
 15 polypeptide of HIV-1 reverse transcriptase and the second p66 subunit polypeptide of HIV-1 reverse transcriptase so as to kill the HIV-1 virus or render the the HIV-1 virus inactive or incapable of infecting cells of a subject, i.e. lack the functions of an infected HIV-1 virus, including human
 20 subjects.

This invention also provides a method of making a pharmaceutical composition comprising an activator of formation of a complex between a first p66 subunit
 25 polypeptide of HIV-1 reverse transcriptase and a second p66 subunit polypeptide of HIV-1 reverse transcriptase which comprises: a) determining whether a compound is an activator of formation of a complex between a first p66 subunit polypeptide of HIV-1 reverse transcriptase and a second p66
 30 subunit polypeptide of HIV-1 reverse transcriptase according

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Moreover, one of skill is not limited to using the yeast cells exemplified in any of the above-described methods herein, but may modify the methods to use other eukaryotic cells, mammalian cells or cell lines such as 298 T cells.

5

Methods of treating a subject infected with HIV-1 include administering any of the above-described pharmaceutical compositions comprising: an inhibitor of the formation of the complex between the p66 subunit polypeptide of HIV-1 reverse transcriptase; an activator of the formation of the complex between the p66 subunit polypeptide of HIV-1 reverse transcriptase and the p51 subunit polypeptide of HIV-1 reverse transcriptase; an inhibitor of formation of a complex between a first p66 subunit polypeptide of HIV-1 reverse transcriptase and a second p66 subunit polypeptide of HIV-1 reverse transcriptase; and/or an activator of formation of a complex between a first p66 subunit polypeptide of HIV-1 reverse transcriptase and a second p66 subunit polypeptide of HIV-1 reverse transcriptase. One of skill will recognize that other pharmaceutical compositions may be administered to a subject infected with HIV-1 in conjunction with the pharmaceutical compositions provided by the methods set forth herein.

25 The invention also provides a pharmaceutical composition comprising an effective amount of any of the above-described inhibitors and a pharmaceutically acceptable carrier. The invention also provides a pharmaceutical composition comprising an effective amount of any of the above-described
30 activators and a pharmaceutically acceptable carrier. In the

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Liquid pharmaceutical compositions which are sterile solutions or suspensions can be utilized by for example, intramuscular, intrathecal, epidural, intraperitoneal or subcutaneous injection. Sterile solutions can also be
 5 administered intravenously. The compounds may be prepared as a sterile solid composition which may be dissolved or suspended at the time of administration using sterile water, saline, or other appropriate sterile injectable medium. Carriers are intended to include necessary and inert binders,
 10 suspending agents, lubricants, flavorants, sweeteners, preservatives, dyes, and coatings.

The inhibitor(s) or activator(s) determined by the methods described above can be administered orally in the form of a
 15 sterile solution or suspension containing other solutes or suspending agents, for example, enough saline or glucose to make the solution isotonic, bile salts, acacia, gelatin, sorbitan monoleate, polysorbate 80 (oleate esters of sorbitol and its anhydrides copolymerized with ethylene oxide) and the
 20 like.

The inhibitor(s) or activator(s) can also be administered orally either in liquid or solid composition form. Compositions suitable for oral administration include solid
 25 forms, such as pills, capsules, granules, tablets, and powders, and liquid forms, such as solutions, syrups, elixirs, and suspensions. Forms useful for parenteral administration include sterile solutions, emulsions, and suspensions.

EXPERIMENTAL DETAILS

FIRST SERIES OF EXPERIMENTS

5 **Materials and Methods**

Bacterial and Yeast Strains

Saccharomyces cerevisiae strain CTY10-5d (MATa ade2 trp1-901 leu2-3, 112 his3-200 gal4-gal80-URA3::lexA-lacZ) contains an integrated GAL1-lacZ gene with the lexA operator (a gift from
10 Stanley Fields, State University of New York, Stony Brook). The yeast strain HF7c contains CYC1-lacZ gene with three copies of the GAL4 responsive UASG 17-mer operator (CLONTECH). Escherichia coli mutator strain XL1-Red (Stratagene) was used for random mutagenesis whereas XL1-Blue
15 (Stratagene) was used to amplify the mutated library. KC8 (CLONTECH), an auxotrophic leuB, trpC and hisB E. coli strain, was used to isolate plasmids from yeast. E.coli strains M15 and BL21 were used to express p66-His and glutathione S-transferase-tagged p51 (GST-p51) respectively
20 (see below).

Yeast Methods

Transformation of yeast and the qualitative β -galactosidase (β -gal) colony lift assay were as published (19).
25 Quantification of protein-protein interactions was determined using the β -gal liquid assay performed on permeabilized yeast grown from three independent transformants using orthonitrophenyl- β -D-galactopyranoside as substrate (19).

30 **Protein Expression and RT Activity**

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Fusion protein expression in yeast was determined by Western blot of lysates with Gal4AD polyclonal antibodies (Upstate Biotechnology, Lake Placid, NY), anti-lexA polyclonal antibodies (Invitrogen) and HIV-1 RT polyclonal (Intracel, Cambridge, MA) or 5B2 monoclonal antibody (20). Immunodetection was with ECL-Plus (Amersham). To measure RT activity, yeast lysates were prepared by glass bead disruption (19) and enzyme activity was determined in exogenous assays (21) and quantified by phosphoimager analysis.

Yeast Shuttle Vectors

pSH2-1 (22) and pLex202-PL (23) express the lexA DNA binding domain (lexA₈₇) and the full-length lexA protein (lexA₂₀₂), respectively. pGBT9 and pAS2-1, both containing the GAL4 DNA binding domain (GAL4 BD), were purchased from CLONTECH. pNLexA allows expression of proteins fused to the N terminus of full-length lexA₂₀₂ (OriGene Technologies, Rockville, MD). pGADNOT (18) and pACTII (24) allow expression of proteins fused to the Gal4 activation domain (GAL4 AD). pACTII also contains the influenza hemagglutinin (HA) epitope tag located between GAL4AD and the polylinker.

Construction of HIV-1 RT Fusions in Yeast Vectors

Constructs and expressed fusion proteins are as described in Fig. 1. The RT sequence for constructing the following expression vectors was amplified from HIV-1 molecular clone pNLenv-1 (containing the HIVNL43 sequence) (25). The p66 amplimers were cloned into the BamHI-SalI sites of pGBT9, pSH2-1, pLex202-PL, pACTII and pGADNOT; the BamHI-XhoI sites

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of pACTII; and the EcoRI-BamHI sites of pNLexA. p51
amplimers were cloned into the BamHI-SalI sites of these
vectors except for cloning into pACTII, where the BamHI-XhoI
sites were used. The HXB2 RT sequence from pHXB2gpt (26) was
5 used to construct p66HXAlaLex202 and p51HXGADNOT.

Construction of HIV-1 RT Deletion Mutants

All p66 deletion mutants were prepared by cloning PCR
amplimers into the BamHI-SalI sites of pSH2-1. Fingers,
10 palm, connection, thumb and RNase H domains of HIV-1 RT are
denoted F, P, C, T and R respectively. pT+C+RSH2-1 (encoding
lexA₈₇-T+C+R) contains RT (from HIVNL43) codons 236-560.
pC+RSH2-1 (encoding lexA₈₇-C+R) contains codons 322-560 while
pRSH2-1 (encoding lexA₈₇-R) comprises codons 425-560. All p51
15 deletion mutants were prepared by cloning of PCR amplimers
into the BamHI-XhoI sites of pACTII. pF+P+T-ACTII (encoding
Gal4AD-HA-F+P+T) includes RT codons 1-325 and pF+P-ACTII
(encoding Gal4AD-HA-F+P) has codons 1-244. p51Δ13ACTII
(encoding Gal4AD-HA-51Δ13) contains RT codons 1-426.
20 p51Δ26GADNOT (encoding Gal4AD-51Δ26) was obtained by random
mutagenesis of p51GADNOT in XL1-Red.

Construction of RT fusions with the L234A Mutation and Random Mutagenesis of p66Ala234Lex202 and Selection of Revertants

25 p66Ala234Lex202 (encoding lexA₂₀₂-Ala-66L234A) was made by
inserting p66 from p6HprotL234A (a gift from Vinayaka Prasad,
Albert Einstein College of Medicine, Bronx NY) into the
BamHI/SalI sites of pLex202-PL. p51234GADNOT (encoding
30 Gal4AD-51L234A) was made by insertion of p51 from
p6HprotL234A into the BamHI-SalI sites of pGADNOT.

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We examined whether the bait fusions encoded by p66SH2-1, p66AlaLex202 and p66NLexA exhibited RT activity in yeast. All three fusion proteins demonstrated high levels of RT activity compared with protein lysates from yeast transformed with an empty vector (data not shown). These data suggest that the p66 fusion proteins are functional and in a conformation consistent with measurable catalytic activity.

10 Heteromeric Interactions of p66 and p51 by Transactivation in the Two-Hybrid System

To test whether the Y2H system could detect the interaction of the p66/p51 heterodimer, we cotransformed yeast reporter strains with plasmids expressing p66DNA BD and p51DNA AD fusion proteins (Table 1). β -gal activity expressed in yeast, which indicates the strength of the interaction between the fusion proteins, was assessed by both qualitative and quantitative assays. The p66 bait fusions expressed from p66SH2-1, p66AlaLex202 and p66NLexA interacted with Gal4AD-p51 domain fusions (Table 1) but not with Gal4AD alone (Table 1). The strongest interactions were observed with p66 baits lexA₂₀₂-Ala-66 and 66-lexA₂₀₂. Moreover, p51 expressed in pACTII gave a stronger signal than p51GADNOT when coexpressed with p66 fusion baits. Despite the stable expression of the p66 fusion protein, lexA₂₀₂-66, no significant interaction with p51 was detected (Fig.1). Moreover, lexA₂₀₂-66 yielded the same weak signal with the empty Gal4AD vector, pGADNOT, indicating that this version of p66 is weakly self-activating even without a partner.

Table 1 Interaction of p66 binding domain fusions with p51 activation domain fusions in the Y2H system

	Constructs	Operator	<u>β-gal activity</u>	
			Colony ^a	Liquid ^a
5	p66SH2-1 + pGADNOT	lexA	-	ND
	p66SH2-1 + pACTII	lexA	-	0.02
	p66SH2-1 + p51GADNOT	lexA	++	0.5
	p66SH2-1 + p51ACTII	lexA	+++	3.5
10	p66AlaLex202 + pGADNOT	lexA	-	ND
	p66AlaLex202 + pACTII	lexA	-	0.04
	p66AlaLex202 + p51GADNOT	lexA	+++	1.6
	p66AlaLex202 + p51ACTII	lexA	+++	7.7
	p66NLexA + pGADNOT	lexA	-	0.06
15	p66NLexA + pACTII	lexA	-	0.04
	p66NLexA + p51GADNOT	lexA	+++	6.6
	p66NLexA + p51ACTII	lexA	+++	25.0
	p66Lex202 + pGADNOT	lexA	+/-	ND
	p66Lex202 + p51GADNOT	lexA	+/-	ND
20	p66GBT9 + pGADNOT ^b	UAS _G	-	ND
	p66GBT9 + p51GADNOT ^b	UAS _G	-	ND

Yeast strain CTY10-5d or ^aHF7c were transformed with plasmids encoding p66 bait and p51 prey fusions. Fusion proteins encoded by plasmids are described in Materials and Methods and Fig. 1.

^aTransformants were lifted onto nitrocellulose and subjected to the β-gal colony lift assay to determine intensities of blue color produced; +++, strong blue in 1 h; ++, blue in 1 h; +/-, weak blue in 3 h; -, white; ND, not done.

30

We also showed that heteromeric interactions between p66 and p51 could be detected in the reciprocal configuration with p51 as either a LexA or Gal4BD fusion and p66 as a Gal4AD fusion (Table 2). The demonstration of heteromeric dimerization of p66 and p51 in different contexts strongly suggests that the interaction is specific. Tests for interaction with five unrelated proteins showed no signal (data not shown), providing further evidence for the specificity of RT heterodimerization in yeast.

40

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Table 2 Interaction of p51 binding domain fusions with p66 activation domain fusions in the Y2H system

5	Constructs	Operator	<u>β-gal activity</u>	
			Colony'	Liquid'
	p51SH2-1 + pGADNOT	lexA	-	0.06
	p51SH2-1 + pACTII	lexA	-	0.05
	p51SH2-1 + p66AlaACTII	lexA	++	1.2
	p51Lex202 + pACTII	lexA	-	0.05
10	p51Lex202 + p66AlaACTII	lexA	+++	3.2
	p51AS2-1 + pACTII [†]	UAS _G	-	ND
	p51AS2-1 + p66AlaACTII [†]	UAS _G	++	ND

15 Yeast strain CTY10-5d or *HF7c were transformed with plasmids encoding p51 bait and p66 prey fusions. Fusion proteins encoded by plasmids are described in Materials and Methods and Fig. 1.

*As defined in Table 1.

†As defined in Table 1.

20

Homomeric Interactions

The interaction of the RT heterodimer p66/p51 has a dissociation constant of 10^{-9} M, whereas the dissociation constants for the p66 and p51 homodimers are 10^{-6} M and 10^{-5} M, respectively (9). We were unable to detect p51 homodimerization when CTY10-5d was cotransformed with either p51SH2-1 or p51Lex202 baits and p51ACTII prey (data not shown). In contrast, p66 homodimerization could be detected when yeast was cotransformed with p66NlexA bait and p66AlaACTII prey (β -gal activity 0.3 Miller units). p66 homodimerization of these two constructs was 100-fold weaker compared to the interaction of p66NlexA with p51ACTII (Table 1). The strength of the interactions observed in vivo are consistent with biochemical data.

35

p66 Domains that Interact with p51

We used the Y2H RT dimerization assay to map the regions of p66 required for binding to p51 (Fig. 2). A series of

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mutants with sequential deletions in the polymerase subdomains were prepared as C-terminal fusions with lexA_{87} . Deletion of the fingers and palm subdomains (lexA_{87} -T+C+R) did not significantly affect binding to Gal4AD-HA-51. A further
5 deletion of the thumb subdomain (lexA_{87} -C+R) resulted in reduced β -gal activity (Fig. 2). Expression of the RNase H domain alone was not sufficient for interaction with p51. This lack of interaction was not attributable to an aberrant
10 RNase H conformation, as lexA_{87} -R also interacted as strongly as lexA_{87} -66 with a cellular protein, diaphorase, that we find interacts with the RNase H domain of RT in the Y2H system (results not shown). None of the bait fusions demonstrated activation of the lacZ reporter gene when coexpressed with Gal4AD-HA alone, excluding the possibility of nonspecific
15 self-activation by the bait fusions (results not shown). These data suggest that the connection and RNase H subdomains of p66 are sufficient for interaction with p51.

The C Terminus of p51 is Required for Interaction with p66

20 It has previously been shown biochemically that deletion of as little as 25 amino acids from the C terminus of p51 can prevent dimerization to p66 (15). To ascertain whether this effect could be observed under physiological conditions in the Y2H system, we constructed a series of C-terminal
25 deletion mutants of p51 prey fusions and assayed interaction with p66 bait. Deletion of 13 amino acids from the C terminus of p51 had little effect (1.8-fold decrease) on dimerization with p66 (Fig. 3). However, deletions of 26 amino acids and greater abrogated RT dimerization, indicating
30 the importance of the C-terminal 26 amino acids of p51 in

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these interactions. These results also suggest that the system faithfully recapitulates the behavior of the enzyme as studied in vitro.

5 **L234A in p66 Subunit Inhibits RT Dimerization**

The Y2H RT dimerization assay would be most useful if it could be applied to the analysis of single amino acid substitutions that affect heteromeric interactions. To test the system, we introduced the L234A primer grip mutation,
10 previously shown biochemically to inhibit p66/p51 association (10), into both RT subunits. The presence of L234A in both p66 and p51 totally inhibited RT dimerization as observed by a 53-fold decrease in the β -gal signal compared to wild-type proteins (Fig. 4). To assess the effect of L234A in
15 individual subunits CTY10d-5 was cotransformed with constructs expressing either p66 mutant bait and wild-type p51 prey, or p66 wild-type bait with p51 mutant prey. Less than a two-fold decrease in the signal compared with wild-type fusions was observed when the L234A mutant p51
20 (Gal4AD-51L234A) was coexpressed with the wild-type fusion lexA_{202} -Ala-66 (Fig. 4). However, a 32-fold inhibition was observed for the interaction of the mutant lex_{202} -Ala-66L234A with wild-type Gal4AD-51. These data suggest that L234A affects dimerization predominantly through p66, as has been
25 previously reported (10). Analysis of fusion protein expression in yeast by Western blot analysis revealed that all fusion proteins, including the L234A mutants, were stably expressed (results not shown).

30 **Second-Site Mutations that Restore Heterodimerization and RT Activity to the p66L234A Mutant**

To gain insight into the mechanism of inhibition of RT dimerization by L234A, we attempted to select for second-site suppressor mutations in p66 that restore dimerization with p51. To select for p66 mutants with restored dimerization, CTY10-5d was cotransformed with a library generated by mutagenesis of p66AlaL234ALex202 and a plasmid expressing either Gal4AD-51 or the Gal4AD-51-L234A mutant. A total of 25,000 colonies from each of two independently mutated libraries were screened. Six and five blue colonies were obtained when lex₂₀₂-Ala-66L234A was cotransformed with Gal4AD-51 and Gal4AD-51-L234A, respectively. CTY10-5d was retransformed with each isolated library plasmid and with either p51HXGADNOT or p51L234AGADNOT; the recovered clones showed restored binding activity with both p51 fusion proteins. Five types of mutations were observed (Table 3). Single amino acid changes in the clones that retained the L234A change included D110G, D186V, W402R and W406R. The remaining three clones had reverted to wild-type at codon 234 (Table 3).

Table 3 Second site mutations in lex_{A202}66HXL234A that restore dimerization to p51

Constructs	No. of Clones	<u>β-gal activity</u>	
		Colony'	Liquid'
Wild Type	NA	+++	3.2
L234A	NA	-	0.1
D110G	NA	+++	6.9
W402R	NA	+++	7.1
W406R	NA	+++	5.8
L234A; D110G	3	+++	1.5
L234A; D186V	1	+/-	0.2
L234A; W402R	3	+++	7.1
L234A; W406R	1	+++	6.1
L234	3	+++	4.7

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Yeast strain CTY10-5d was cotransformed with p51HXGADNOT and various clones expressing *lexA*₂₀₂-Ala-66HX fusions with mutations in p66 as indicated. NA, not applicable.

*As defined in Table 1.

5 *As defined in Table 1.

Two of the changes are at the catalytically essential aspartyl residues D110 and D186. These residues are not located at the dimer interface, and mutations at these
10 residues result in an inactive RT (28) (Fig. 5). A variant p66 containing D110G alone, without L234A, gave a 2-fold stronger β -gal signal than wild-type p66 for heterodimerization and was 4.6-fold stronger compared with clones containing both L234A and D110G. Partial restoration
15 of dimerization by D110G suggests that conformational changes at the active site compensate for structural changes mediated by L234A.

The second set of mutations, W402R and W406R, are located at
20 the dimer interface (Fig. 5) in a tryptophan repeat region which is highly conserved among HIV-1, HIV-2 and closely related simian immunodeficiency virus RTs (29). In the L234A genetic background, these mutations resulted in a dramatic increase in the β -gal signal over the parent and yielded a
25 2-fold higher signal for heterodimerization compared with wild-type RT fusions (Table 3). W402R and W406R in a wild-type genetic background had the same enhanced β -gal activity as the restored mutants (Table 3). Therefore, the mutations in the tryptophan repeat motif may enhance the
30 interaction with GAL4AD-51 independently of the L234A mediated defect.

To confirm that the second-site mutations could restore

heterodimerization to the L234A parent in an alternative assay, we examined the binding of these p66 mutants to p51 in vitro. Bacterial lysates containing GST-p51 or wild-type and mutant p66-His were incubated together, and heterodimers were
5 captured on Glutathione Sepharose 4B beads. As expected, wild-type p66 dimerized with GST-p51 whereas the p66L234A mutant did not (Fig. 6A). Restoration of dimerization by D110G, W402R or W406R in the L234A parent was observed (Fig. 6A), thus confirming our observations in the Y2H assay.

10

To determine whether restoration of heterodimerization was associated with enhanced DNA polymerase activity, heterodimers eluted from beads were assayed for RT activity (Fig 6C). GST-p51 had significant background activity
15 compared with wild-type enzyme. The enzyme resulting from incubation with p66L234A had the same background activity. As expected, heterodimers comprising p66L234A containing the active site mutation D110G also had only background activity. Interestingly, both W402R and W406R mutations not only
20 restored heterodimerization to the L234A parent but also increased RT activity, even above levels of the wild-type control (Fig. 6C).

Discussion

25 In this study we have shown that fusions of p66 and p51 can be stably expressed in yeast and can heterodimerize in reciprocal configurations. The presence of spacers in the form of alanine or an HA tag may have been an important aspect for stronger interactions in the Y2H assay. Moreover,
30 we have validated the Y2H assay by comparing previously

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described effects of p51 deletions and the L234A substitution on heterodimerization. We have also shown how this assay can further the study of the HIV-1 RT structure-function by the identification of second-site mutations that restore RT
5 dimerization.

The palm, connection, and RNase H domains of p66 make major contacts with p51. An indication that the palm region is important is the destabilization of the p66/p51 heterodimer
10 by the nonnucleoside RT inhibitor 2', 5'-bis-O-(tert-butylidimethylsilyl)-3'-spiro-5"-4"-amino-1",2"-oxothiole-2",2"-dioxide)]-b-D- pentofuranosyl (TSAO) by its interaction between the palm subdomain of p66 and the β 7- β 8 loop in the fingers subdomain of p51 (30, 31). Preliminary
15 tests of the addition of TSAO to our in vitro binding assays confirm the ability of the drug to reduce heterodimerization (data not shown). Tests of the related drug TSAOe³T showed a more modest destabilization only detectable in the presence of denaturants (31). Deletion mapping of the p66 domains
20 required for interaction with p51 suggests that the presence of the connection and RNase H domains are sufficient for interaction with p51 in the Y2H system. It is surprising that the deletion of the palm domain had little effect on binding to p51 as this p66 subdomain provides a major contact
25 with p51 (9); however, the connection and RNase H domains may provide a sufficient surface for saturating the signal in yeast.

Truncation of the C terminus of p51 revealed that a 13-amino
30 acid deletion had little effect on dimerization with p66, but

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a deletion of 26 amino acids abrogated heterodimerization as seen in the Y2H assay. These data are consistent with previous in vitro studies (15). All C-terminal truncation mutants were stably expressed in yeast, excluding the possibility of decreased expression affecting the signal. It is possible that these C-terminal residues may have a direct role in dimerization; or the deletion of these residues may effect the structural integrity or correct positioning of the structural elements α -L and β -20 (5,15). These elements contain the tryptophan repeat motif, which has been proposed to play an important role in HIV-1 dimerization (29, 32).

We have shown that the L234A substitution inhibits RT dimerization in yeast most dramatically when present on the p66 subunit of HIV-1 RT, as previously seen in vitro (10). L234A is located in the primer grip region of p66 (5) and is highly conserved among avian, primate and murine RTs (33). To help determine the mechanism by which L234A affects heterodimerization, we selected for second-site mutations restoring p66/p51 association. Aside from clones which had reverted to the wild-type L234, we observed two classes of mutants: those with alterations either in the tryptophan repeat or in the polymerase active site (Fig. 5). Both classes of suppressors were also shown to restore binding of the mutant p66 subunit to p51 as measured in an in vitro binding assay (Fig. 6A). L234A is not at the dimer interface, and it has been proposed that it affects dimerization by indirectly affecting contacts between P95 in the palm of p66 with residues in the β 7- β 8 loop of p51 (11). The mutations W402R and W406R are distant from this region, being located

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in the connection subdomain which contacts the p51 connection domain in the heterodimer. The appearance of a basic residue in both codon 402 and 406 suggests a charge interaction with an acidic residue in p51 or alternatively an increase in electrostatic potential between the surfaces at the connection domain interface.

The recovery of second-site suppressor mutations at the catalytically essential aspartyl residues suggests that there is a relationship between dimerization and active site residues. Neither D186V nor D110G make obvious contacts with L234A, although both are in the same palm subdomain (Fig. 5)(2). Interaction between the NNRTI binding site, which includes L234, and the RT catalytic site has been suggested by both structural and enzymatic data explaining the mechanism of resistance to NNRTIs (34, 35). The D110G or D186V changes would probably result in loss of one of the two magnesium ions bound to the active site (36). A loss of chelated magnesium in addition to a glycine change at 110 may lead to increased flexibility in that region, thus affecting dimerization. Determination of the crystal structure of the D110G RT mutant will help resolve these issues.

Heterodimerization of HIV-1 has been suggested as a target for chemotherapeutic intervention (7). To date, there are no HIV-1 RT dimerization inhibitors being used in the clinic. Nevertheless, there are several reports of HIV-1 and HIV-2 RT dimerization inhibitors based on peptides representing the conserved tryptophan repeat region of RT (32, 37). These peptides have been shown to prevent the association of

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p66/p51 (32) and have demonstrable in vitro anti-HIV-1 activity (37). TSAO has been shown to destabilize the p66/p51 heterodimer and may represent a nonpeptide RT dimerization inhibitor (30). In preliminary tests of this
5 drug for its effects on heterodimerization in the Y2H system, we saw no inhibition of β -gal activity (data not shown). However, the possibility that the drug is not taken up by yeast cannot be ruled out. The availability of a Y2H assay for RT dimerization will facilitate the screening for other
10 such inhibitors of this process according to the methods set forth herein.

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REFERENCES FOR FIRST SERIES OF EXPERIMENTS

1. di Marzo Veronese, F., Copeland, T. D., DeVico, A. L., Rahman, R., Oroszlan, S., Gallo, R. C. & Sarngadharan, M. G. (1986) Science 231, 1289-1291.
5
2. Rodgers, D. W., Gamblin, S. J., Harris, B. A., Ray, S., Culp, J. S., Hellmig, B., Woolf, D. J., Debouck, C. & Harrison, S. C. (1995) Proc. Natl. Acad. Sci. USA 92, 1222-1226.
10
3. Ren, J., Esnouf, R., Garman, E., Somers, D., Ross, C., Kirby, I., Keeling, J., Darby, G., Jones, Y., Stuart, D., et al. (1995) Nat. Struct. Biol. 2, 293-302.
15
4. Huang, H., Chopra, R., Verdine, G. L. & Harrison, S. C. (1998) Science 282, 1669-1675.
5. Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Jr., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., et al. (1993) Proc. Natl. Acad. Sci. USA 90, 6320-6324.
20
6. Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A. & Steitz, T. A. (1992) Science 256, 1783-1790.
25
7. Restle, T., Muller, B. & Goody, R. S. (1990) J. Biol. Chem. 265, 8986-8988.
- 30 8. Becerra, S. P., Kumar, A., Lewis, M. S., Widen, S. G.,

-103-

- Abbotts, J., Karawya, E. M., Hughes, S. H., Shiloach, J. & Wilson, S. H. (1991) *Biochemistry* 30, 11707-11719.
- 5 9. Wang, J., Smerdon, S. J., Jager, J., Kohlstaedt, L. A., Rice, P. A., Friedman, J. M. & Steitz, T. A. (1994) *Proc. Natl. Acad. Sci. USA* 91, 7242-7246.
- 10 10. Ghosh, M., Jacques, P. S., Rodgers, D. W., Ottman, M., Darlix, J. L. & Le Grice, S. F. (1996) *Biochemistry* 35, 8553-8562.
11. Wohrl, B. M., Krebs, R., Thrall, S. H., Le Grice, S. F. J., Scheidig, A. J. & Goody, R. S. (1997) *J. Biol. Chem.* 272, 17581-17587.
- 15 12. Goel, R., Beard, W. A., Kumar, A., Casas-Finet, J. R., Strub, M. P., Stahl, S. J., Lewis, M. S., Bebenek, K., Becerra, S. P., Kunkel, T. A., et al. (1993) *Biochemistry* 32, 13012-13018.
- 20 13. Divita, G., Restle, T. & Goody, R. S. (1993) *FEBS Lett.* 324, 153-158.
- 25 14. Debyser, Z. & De Clercq, E. (1996) *Protein Sci.* 5, 278-286.
15. Jacques, P. S., Wohrl, B. M., Howard, K. J. & Le Grice, S. F. (1994) *J. Biol. Chem.* 269, 1388-1393.

-104-

16. Fields, S. & Song, O. (1989) Nature (London) 340, 245-246.
17. Kalpana, G. V. & Goff, S. P. (1993) Proc. Natl. Acad. Sci. USA 90, 10593-10597.
18. Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V. & Goff, S. P. (1993) Cell 73, 1067-1078.
19. Rose, M. D., Winston, F. & Hieter, P. (1990) Methods in Yeast Genetics: A Laboratory Course Manual (Cold Spring Harbor Laboratory Press, New York),
20. Szilvay, A. M., Nornes, S., Haugan, I. R., Olsen, L., Prasad, V. R., Endresen, C., Goff, S. P. & Helland, D. E. (1992) J. Acquired Immune Defic. Syndr. 5, 647-657.
21. Telesnitsky, A., Blain, S. & Goff, S. P. (1995) Methods Enzymol. 262, 347-362.
22. Hanes, S. D. & Brent, R. (1989) Cell 57, 1275-1283.
23. Ruden, D. M., Ma, J., Li, Y., Wood, K. & Ptashne, M. (1991) Nature (London) 350, 250-252.
24. Legrain, P., Dokhelar, M. C. & Transy, C. (1994) Nucleic Acids Res. 22, 3241-3242.
25. Maldarelli, F., Martin, M. A. & Strebel, K. (1991) J. Virol. 65, 5732-5743.

26. Ratner, L., Haseltine, W., Patarca, R., Livak, K. J.,
Starcich, B., Josephs, S. F., Doran, E. R., Rafalski,
J. A., Whitehorn, E. A., Baumeister, K., et al. (1985)
Nature (London) 313, 277-284.
- 5 27. Maier, G., Dietrich, U., Panhans, B., Schroder, B.,
Rubsamen-Waigmann, H., Cellai, L., Hermann, T. &
Heumann, H. (1999) Eur. J. Biochem. 261, 10-18.
- 10 28. Larder, B. A., Purifoy, D. J., Powell, K. L. & Darby,
G. (1987) Nature (London) 327, 716-717.
29. Baillon, J. G., Nashed, N. T., Kumar, A., Wilson, S.
H. & Jerina, D. M. (1991) New Biol. 3, 1015-1019.
- 15 30. Harris, D., Lee, R., Misra, H. S., Pandey, P. K. &
Pandey, V. N. (1998) Biochemistry 37, 5903-5908.
- 20 31. Sluis-Cremer, N., Dmitrienko, G. I., Balzarini, J.,
Camarasa, M. J. & Parniak, M. A. (2000) Biochemistry
39, 1427-1433.
- 25 32. Divita, G., Restle, T., Goody, R. S., Chermann, J. C.
& Baillon, J. G. (1994) J. Biol. Chem. 269,
13080-13083.
- 30 33. Georgiadis, M. M., Jessen, S. M., Ogata, C. M.,
Telesnitsky, A., Goff, S. P. & Hendrickson, W. A.
(1995) Structure (London) 3, 879-892.

34. Esnouf, R., Ren, J., Ross, C., Jones, Y., Stammers, D.
& Stuart, D. (1995) Nat. Struct. Biol. 2, 303-308.
35. Spence, R. A., Kati, W. M., Anderson, K. S. & Johnson,
5 K. A. (1995) Science 267, 988-993.
36. Patel, P. H., Jacobo-Molina, A., Ding, J., Tantillo,
C., Clark, A. D., Jr., Raag, R., Nanni, R. G., Hughes,
S. H. & Arnold, E. (1995) Biochemistry 34, 5351-5363.
10
37. Morris, M. C., Robert-Hebmann, V., Chaloin, L., Mery,
J., Heitz, F., Devaux, C., Goody, R. S. & Divita, G.
(1999) J. Biol. Chem. 274, 24941-24946.
- 15 38. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24,
946-950.
39. Merrit, E. A. & Bacon, D. J. (1997) Methods Enzymol.
277, 505-524.
- 20 40. Kabsch, W. & Sander, C. (1983) Biopolymers 22,
2577-2637.

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SECOND SERIES OF EXPERIMENTS

Nonnucleoside reverse transcriptase inhibitors (NNRTIs) are allosteric inhibitors of the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT). Yeast grown in
5 the presence of many of these drugs exhibited dramatically increased association of the p66 and p51 subunits of the HIV-1 RT as reported by a yeast two-hybrid assay. The enhancement required drug binding by RT; introduction of a drug-resistance mutation into the p66 construct negated the
10 enhancement effect. The drugs could also induce heterodimerization of dimerization defective mutants. Coimmunoprecipitation of RT subunits from yeast lysates confirmed the induction of heterodimer formation by the drugs. In vitro binding studies indicate that NNRTIs can
15 bind tightly to p66 but not p51, and then mediate subsequent heterodimerization. This study demonstrates a novel effect of NNRTIs on the assembly of RT subunits.

The human immunodeficiency virus type 1 (HIV-1) reverse
20 transcriptase (RT) catalyzes the conversion of genomic RNA into double-stranded proviral DNA after cell entry (1), utilizing the RNA- and DNA-dependent polymerase and ribonuclease H (RNase H) activities of the enzyme. The HIV-1 RT is an asymmetric dimer consisting of p66 and p51
25 polypeptides (2, 3). The p51 subunit contains the identical N-terminal sequences as p66, but lacks the C-terminal RNase H domain. The structure of the HIV-1 RT has been elucidated by X-ray crystallography in several forms including the unliganded enzyme (4), in complex with nonnucleoside reverse
30 transcriptase inhibitors (NNRTIs) (5, 6) and bound to

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template-primer with (7) or without dNTP substrate (8). The polymerase domain of the p66 subunit resembles a right hand and contains the fingers, palm, thumb and connection subdomains, with the latter acting as a tether between the
5 polymerase and RNase H regions (5, 8). Although p51 has the same polymerase domains as p66, the relative orientations of these individual domains differ markedly (5, 8). Structural analysis reveals three major contacts between p66 and p51, with most of the interaction surfaces being hydrophobic (9,
10 10).

NNRTIs are chemically diverse, largely hydrophobic compounds which comprise over 30 different classes (11, 12). NNRTIs do not require intracellular metabolism for activity, are
15 noncompetitive inhibitors of RT activity with respect to dNTP substrate and template/primer, and are relatively noncytotoxic (11). NNRTIs bind to a hydrophobic pocket close to but distinct from the polymerase active site in the p66 subunit (13, 14) and inhibit enzyme activity by mediating
20 allosteric changes in the RT (15, 16). Initial clinical use of NNRTIs as monotherapy and selection of drug-resistant variants in cell culture results in the rapid emergence of highly drug-resistant variants due to single amino acid changes (17, 18) in the NNRTI binding pocket that directly
25 affect drug binding (13, 14). The NNRTIs currently approved for use in highly active antiretroviral therapy include nevirapine (19), delavirdine (20) and efavirenz (21).

We have previously shown that HIV-1 RT heterodimerization can
30 be effectively monitored in the yeast two-hybrid (Y2H) system

Construction of HIV-1 RT fusions in yeast expression vectors

The construction of p66SH2-1, p51SH2-1, p66GADNOT, p51GADNOT and p51ACTII which express the wild type p66 and p51 fusion proteins *lexA₈₇-66*, *lexA₈₇-51*, *Gal4AD-66*, *Gal4AD-51* and *Gal4AD-HA-51*, respectively were as described previously (22). p66L234ASH2-1 (encoding *lexA₈₇-66L234A*) was made by cloning the PCR amplification product from the RT region of p66AlaL234ALex202 (22) into the *Bam*HI-*Sal*I sites of pSH2-1. p51234ACTII (encoding *Gal4AD-HA-51L234A*) was constructed by subcloning the p51 *Bam*HI-*Sal*I fragment from p51234GADNOT (22) into pACTII. p66W401ASH2-1 (encoding *lexA₈₇-66W401A*), p51W401AACTII (encoding *Gal4AD-HA-51W401A*) and p51W401AGADNOT (encoding *Gal4AD-51W401A*) were made by PCR amplification of the RT region from plasmid pALRT-78S(A402) (a gift from John McCoy) and cloned into the *Bam*HI-*Sal*I sites of pSH2-1, pGADNOT or the *Bam*HI-*Xho*I sites of pACTII. p66Y181CSH2-1 containing the Y181C mutation in p66 of the *lexA₈₇-66* fusion protein was prepared by site-directed mutagenesis using the Gene Editor Kit (Promega, Madison, WI) according to the manufacturer's protocol.

Construction of HIV-1 RT fusions in bacterial expression vectors

Wild-type and p66 mutants (either L234A or W401A) were cloned into the *Sph*I-*Bgl*III site of pQE-70 (Qiagen, Chatsworth, CA) (22). Glutathione S-transferase-tagged p51 (GST-p51) and mutants containing either the W401A or L234A substitutions were constructed by cloning the p51 encoding fragments into the *Bam*HI-*Sal*I site of pGEX5X-3 (Amersham Pharmacia Biotech)

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(22).

Y2H RT heterodimerization assays for measuring effect of NNRTIs on b-gal activity.

5 CTY10-5d transformed constructs expressing p66 bait and p51 prey fusions were grown overnight to stationary phase in synthetic complete medium without histidine and leucine and containing 2% glucose (SC-His-Leu). 2.5 ml of media with or without drug were inoculated with 0.0125 - 0.25 OD₆₀₀ units of
10 CTY10-5d. Yeast were grown with aeration at 30°C to OD₆₀₀ = 0.5. The equivalent of 1 OD₆₀₀ unit was pelleted for each treatment and subjected to a quantitative b-gal liquid assay.

15 **Coimmunoprecipitation of p66 and p51 in yeast lysates.**

Cultures (30 ml) containing no drug, efavirenz or UC781 and 0.1 OD₆₀₀ units/ml of CTY10-5d expressing p66 bait and p51 prey fusions were grown in SC-His-Leu to OD₆₀₀ = 0.5 at 30°C. Cells were normalized to 12 OD₆₀₀ units and washed with 10 ml
20 of TE (10 mM Tris pH 7.5; 1 mM EDTA) buffer. Preparation of protein extracts and immunoprecipitation were as previously described (30) except for the use of anti-HA.11 monoclonal antibodies (clone 16B12; Covance, Princeton, NJ) and Protein G-PLUS agarose beads (Santa Cruz Biotechnology; Santa Cruz,
25 CA). Samples were resolved by SDS-PAGE. The lexA₈₇-66 fusion protein was probed using monoclonal antibodies 7E5 which specifically detects p66 (31).

In vitro heterodimerization in the presence of NNRTIs

30 The heterodimerization of bacterially expressed wild-type

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p66-His and GST-p51 (or mutants) was assessed in bacterial lysates as described previously (22). To determine the capacity of efavirenz to bind to a particular RT subunit, 500 μ l reactions in lysis buffer (without NP-40) (22), 5 μ g of
 5 p66-His, 5 μ g GST-p51 or no recombinant protein (total protein concentration was 0.8 μ g/ml in each reaction) were incubated overnight at 4°C with increasing concentrations of efavirenz. Lysates were washed 4 times with lysis buffer using a centricon-YM-50 filter device (Millipore Corporation,
 10 Bedford, MA) to remove unbound drug. 5 μ g of the corresponding RT subunit was applied to the washed lysates (in 500 μ l) and incubated for 1.5 hr at 4°C. Heterodimers were captured onto beads (22), resolved by SDS/PAGE and detected using RT antibodies (monoclonal antibody 5B2) (31).

15

Results

Enhancement of b-gal activity by NNRTIs

To test the effects of NNRTIs on the association of the RT polypeptides we used a yeast genetic assay that measures RT
 20 heterodimerization (22). In this assay yeast expressing the p66 subunit of the HIV-1 RT fused to the C-terminus of lexA₈₇ (lexA₈₇-66) and the p51 subunit fused to the Gal4AD (Gal4AD-51) constitutively interact, resulting in the activation of
 25 the expression from an integrated Lac Z reporter gene. To test for the effects of the NNRTIs on this interaction, 10 drugs representing 7 different NNRTI classes were added to the culture medium during growth of the yeast and b-gal levels were determined. Of the 10 NNRTIs tested, 9
 30 demonstrated a dramatic concentration-dependent enhancement

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of b-gal activity compared to cells not treated with drug (Figs 7A and 7B). No significant toxicity, as determined by the growth rate, was observed for the drug concentrations tested compared to untreated controls (results not shown).

5 Efavirenz was the most potent of the compounds, mediating a 40-fold increase in b-gal activity at the highest drug concentration tested (Fig. 7A). The carboxanilide UC781 was the second most potent drug, followed by UC10 and a quinoxaline, HBY 097 (Figs 7A and 7B). The remainder of the

10 NNRTIs were less potent but still displayed 8 - 10 fold increases in b-gal activity at the highest concentrations tested (Figs 7A and 7B). In contrast delaviridine was devoid of b-gal enhancing activity (Fig. 7A).

15 **Enhancement of b-gal activity by NNRTIs is specific for RT heterodimerization**

The specificity of the b-gal enhancement by NNRTIs was investigated. Yeast transformed with the empty vectors pSH2-1 and pGADNOT, which express lexA₈₇ and Gal4AD, respectively

20 were treated with serial dilutions of the most potent b-gal enhancing drug, efavirenz. We observed no increase in b-gal activity even in the presence of 15 μ M of drug (data not shown). The capacity of efavirenz to enhance b-gal activity of several unrelated protein-protein interaction pairs,

25 including moloney murine leukemia virus reverse transcriptase with elongation factor release factor 1 (M.O., unpublished), was also examined and no enhancement or inhibition of b-gal activity was observed.

30 The Y181C mutation in the p66 subunit of the HIV-1 RT confers

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more than a 100-fold increase in resistance to nevirapine (17). This mutation directly affects drug binding (13, 14). To further establish the specificity of the b-gal enhancement by NNRTIs, Y181C was introduced into the plasmid encoding the
 5 lexA₈₇₋₆₆ fusion protein. Yeast were cotransformed with various pairs of plasmids and grown in the presence of nevirapine. The presence of the Y181C change in the p66 bait totally negated the enhancement effect by nevirapine (Fig. 8). In contrast, a significant level of b-gal enhancement
 10 was still retained in the presence of efavirenz (results not shown), consistent with the very low level of resistance conferred by Y181C to this drug. These data provide compelling evidence that the b-gal enhancement effect is due to a specific interaction of nevirapine with the p66 subunit
 15 of the HIV-1 RT.

NNRTIs can enhance b-gal activity of dimerization defective mutants

Previous studies have shown that the L234A mutation in HIV-1
 20 RT abrogates RT dimerization (22, 32). Other studies on the role of the tryptophan repeat motif (codons 398-414), present in the connection subdomains of both subunits, showed that the W401A mutation also diminishes RT dimerization in the Y2H assay (G.T. unpublished data). We investigated the effect of
 25 the NNRTIs, efavirenz and UC781 on the b-gal enhancement effect on these dimerization defective RT mutants. Interestingly, yeast treated with efavirenz and expressing the W401A change in one or both subunits showed a dramatic increase in b-gal activity compared to no drug (Fig. 9A). b-
 30 gal activity in yeast expressing the W401A mutation in both

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expressing dimerization defective mutants to coimmunoprecipitation. Yeast expressing both p66 bait and p51 prey fusions containing the W401A mutation (lexA₈₇-66W401A and Gal4AD-HA-51W401A) or the L234A change (lexA₈₇-66L234A and Gal4AD-HA-51L234A) were grown in the presence of efavirenz (1.6 μ M), UC781 (16 μ M) or no drug. Hemagglutinin (HA) antibodies were used to immunoprecipitate the p51 prey and the presence of any bound p66 bait was then detected using anti-p66 specific antibodies. For coimmunoprecipitation of the p66 bait fusions, samples were divided into two with one part processed without added NNRTI while the other was maintained in drug at the same concentration used during growth of yeast. Yeast grown in the absence of drug was also processed without drug or in the presence of 1.6 μ M of efavirenz. A clear increase in the amount of lexA₈₇-66W401A and lexA₈₇-66L234A associated with the p51 prey was observed for yeast grown in the presence of efavirenz compared to no drug (Figs 4A and 4B). Similar experiments with yeast grown in UC781 revealed heterodimer formation for yeast expressing the W401A mutant but not for the L234A mutant; these data corresponding to the levels of b-gal activity in the cells (Figs 4A and 4B).

No significant difference in the amount of coimmunoprecipitated p66 bait in the absence or presence of drug was observed indicating that the heterodimer was stable under the conditions of the assay. Interestingly, there was significantly more heterodimer present in yeast lysates obtained from cells grown in the absence of drug to which efavirenz was added during the coimmunoprecipitation

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procedure for the W401A mutant (Fig. 10A). These data suggest that some heterodimer formation could occur in vitro. Levels of mutant p66 bait and p51 prey fusions present in the original lysate from yeast grown in the absence and presence of drug were similar indicating that the increase in coimmunoprecipitated p66 bait in the presence of drug was not due to increased levels of fusion proteins. It is clear from these experiments that NNRTIs tested do act by inducing heterodimerization of p66 and p51 in the Y2H assay and that the increased dimer formation correlates with the increase in b-gal activity.

Efavirenz enhances the association of wild-type and mutant p66 and p51 in lysates in vitro

To explore whether NNRTIs could enhance dimerization in vitro, bacterial lysates containing either p66-His or GST-p51 were prepared and combined in the presence of increasing concentrations of efavirenz. In the absence of inhibitor a small amount of dimer was present as indicated by detectable amounts of p66-His. A concentration dependent increase in dimer formation was observed in the presence of increasing concentrations of efavirenz (Fig. 11). The enhancement effect of efavirenz on the L234A and W401A mutants was also assessed. Bacterial lysates separately expressing p66L234A-His and GST-p51L234A or p66W401A-His and GST-p51W401A were combined as above and incubated in the presence of increasing concentrations of efavirenz. A significant increase in dimer formation was observed in the presence of a 10-fold molar excess of efavirenz for the W401A mutant (Fig. 11). A 100-fold molar excess of efavirenz over RT was required to induce

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detectable enhancement of dimerization of the L234A mutant (Fig. 11). These data are consistent with the coimmunoprecipitation experiments and indicate that the enhancement of dimerization by efavirenz is due to its
5 specific interaction with the HIV-1 RT and not dependent on the fusion proteins used in the Y2H assay nor on components present in the yeast cells in vivo.

**Other NNRTIs enhance heterodimerization of RT subunits in
10 vitro**

We extended our in vitro study by testing the remaining NNRTIs for their capacity to enhance the dimerization of GST-p51 and p66-His in vitro. Consistent with our Y2H data we observed that efavirenz was the most potent enhancer of
15 dimerization. The relative in vitro potencies of the other NNRTIs correlated well with their b-gal enhancing effect in yeast (Figs 7 and 12). In contrast, UC781 and UC10 were poor dimerization inducers in bacterial lysates compared with their b-gal enhancing activities. The low dimerization
20 enhancement activity of these drugs may be a function of both their poor solubility and the conditions of the in vitro assay (which was performed at 4°C). In contrast, the conditions of the yeast assay, which was carried out at 30°C with agitation, may have facilitated solubilization of UC781
25 and UC10. Interestingly, delavirdine was also inactive in vitro indicating that the lack of effect in yeast was not a result of the inability of this drug to penetrate the cells.

**Efavirenz enhances heterodimerization by binding to p66-His
30 but not GST-p51**

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To help elucidate the mechanism by which efavirenz enhances heterodimerization we assessed whether this drug could bind to either p66-His or GST-p51. Bacterial lysates expressing p66-His, GST-p51 or no recombinant protein were preincubated
5 in the absence or presence of increasing concentrations of efavirenz. Unbound drug was removed from the lysates by a series of washes and the presence of any remaining drug was assayed by the addition of the cognate RT subunit. p66-His and GST-p51 was added to a washed mock bacterial lysate to
10 assess the efficiency of efavirenz removal. When p66-His was preincubated with efavirenz we observed enhancement of dimerization with subsequently added GST-p51 at all drug concentrations (Fig. 13). This enhancement was similar to controls where p66-His and GST-p51 were simultaneously
15 combined with various drug concentrations (Fig. 12). A 100-fold reduction in the potency of heterodimerization compared to p66-His preincubated with efavirenz was observed in the washed mock bacterial lysate (Fig. 13). GST-p51 preincubated with drug, washed and then subjected to the functional
20 heterodimerization assay displayed the same pattern of heterodimerization observed for the drug-treated mock bacterial lysate. These data indicate that efavirenz binds tightly to p66-His but not GST-p51 and that this binding then promotes heterodimerization with subsequent added GST-p51.

25

Discussion

This study reports a previously undescribed property of certain NNRTIs - their capacity to enhance heterodimerization of the p66 and p51 subunits of the HIV-1
30 RT. This effect was observed both in the Y2H system,

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detecting dimerization of p66 and p51 using b-gal activity as a readout, and confirmed in coimmunoprecipitation experiments. The phenomenon was also observed in vitro using bacterially expressed GST-p51 and p66-His showing that it is not specific to yeast. NNRTIs were also able to induce the dimerization of the interaction defective mutants L234A and W401A. Furthermore, efavirenz can bind tightly to p66-His and then subsequently promote heterodimerization. The data indicate that NNRTIs have properties similar to conventional CIDs in their capacity to enhance the interaction between two proteins. As the interaction between p66 and p51 occurs naturally and the effect of the NNRTIs is to enhance this interaction then these small molecules are best described as chemical enhancers of dimerization.

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Correlation between in vitro and in vivo enhancement of heterodimerization by NNRTIs

The most potent b-gal enhancing NNRTIs in the Y2H RT dimerization assay were efavirenz, UC781 and HBY 097. These drugs are second generation NNRTIs that are also extremely potent inhibitors of HIV-1 replication in vitro (21, 25, 27). Efavirenz and UC781 differ from the other NNRTIs in that they bind very tightly to the RT heterodimer and exhibit very slow dissociation rates (k_{off}) (34, 35). The tight binding properties of efavirenz and UC781 may in part have contributed to their potency as enhancers of heterodimerization in yeast. There was generally a very good correlation between the relative potency in inducing dimerization of the NNRTIs in vitro and in yeast, with the exception of UC781 and UC10.

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Relationship between drug induced enhancement of dimerization, structural changes in the HIV-1 RT and RT inhibitory activity.

NNRTIs bind in a hydrophobic pocket at the base of the p66 thumb subdomain which is proximal to (~ 10 Å), but distinct from the polymerase active. It is clear that the size of the NNRTI binding pocket is small compared to the extensive dimer interface (Fig. 14). No strong correlation was found between the extent of the p66/p51 interface (36, 37) in the structures of the HIV-1 RT in complex with several NNRTIs and the drug concentration mediating a 5-fold enhancement of b-gal activity. Thus, the NNRTI effect on heterodimerization is not a simple function of the surface area buried at the interface, and NNRTIs may affect dimerization by other mechanisms in addition to modulating the extent of the contacts. The position of the drug in the pocket and the degree of NNRTI interaction with the p51 subunit were found to vary significantly among the different RT/NNRTI complexes (Fig. 9), and the changes in the vicinity of the bound NNRTIs may also play a role in heterodimer formation.

Binding of efavirenz to RT is accompanied by conformational changes in the binding pocket region, and these changes (including at Leu234) (38), may also influence dimer formation. Delavirdine is the longest NNRTI inhibitor and a portion of it protrudes outside the NNRTI binding site causing the largest distortion of the p66 subunit of any of the NNRTIs studied to date (39). Delavirdine binds the furthest away from the p66/p51 interface (closest distance between delavirdine and p51 is 5.1 Å compared to 3.8 Å for

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UC781) (Fig. 15). The unique characteristics of the interaction of delavirdine with HIV-1 RT suggest that this NNRTI may bind to p66 in a distinctive way that does not favor the enhancement of dimerization.

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The relationship between the RT inhibitory activity of the NNRTIs in an exogenous RT assay (50% inhibitory concentration) and the concentration of drug that mediates a 5-fold increase in b-gal activity in the Y2H assay was compared. Efavirenz was the most potent in both assays while UC38 was the least active (results not shown). Examination of the data revealed a fair correlation ($r = 0.6$) between these two parameters suggesting a relationship between the b-gal enhancement effect and RT inhibitory activity in vitro.

Potential mechanisms for NNRTI enhancement of dimerization

How might the NNRTIs enhance heterodimerization? One possible model involves NNRTI binding directly to the p66 monomer. Drug binding to monomeric p66 may stabilize a conformation that is more conducive to heterodimer formation, and a more potent NNRTI may effectively increase the concentration of p66 in a conformation that promotes dimerization. Alternatively, efavirenz may cause the p66 monomer to have a conformational flexibility that allows this subunit to more readily undergo structural changes necessary for dimerization. A second model would entail NNRTIs binding only to the heterodimer and as a consequence stabilizing the dimer. The binding could shift the equilibrium toward the dimer. The data suggest that efavirenz binds tightly to p66.

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However, as bacterially expressed p66 comprises a population of monomers and homodimers it is unclear whether GST-p51 is binding directly to monomeric p66 complexed with drug or is exchanging with one p66 subunit in the drug bound homodimer.

5 Elucidation of the exact mechanism of NNRTI induced enhancement of dimerization will require further studies.

The findings may have biological significance in terms of effects on virus replication. Drug binding to p66 could
10 potentially modulate the interaction between Pr160^{GagPol} precursors which may affect regulation of HIV-1 protease-specific cleavage of this polyprotein. Further, the Y2H RT dimerization assay can potentially be used to screen for NNRTIs with the capacity to bind and mediate the appropriate
15 conformational changes in the p66 subunit that results in enhanced binding to p51. It is possible that novel allosteric inhibitors of RT may be selected using this assay.

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REFERENCES FOR SECOND SERIES OF EXPERIMENTS

31. Goff, S. P. (1990) *J. Acquir. Immune Defic. Syndr.* **3**, 817-831.
32. di Marzo Veronese, F., Copeland, T. D., DeVico, A. L.,
5 Rahman, R., Oroszlan, S., Gallo, R. C. & Sarngadharan, M. G. (1986) *Science* **231**, 1289-1291.
33. Le Grice, S. F. J. (1993) in *Reverse Transcriptase*, eds. Skalka, A. M. & Goff, S. P. (Cold Spring Harbor Laboratory Press, Plainview), pp. 163-191.
- 10 34. Hsiou, Y., Ding, J., Das, K., Clark, A. D., Jr., Hughes, S. H. & Arnold, E. (1996) *Structure* **4**, 853-860.
35. Kohlstaedt, L. A., Wang, J., Friedman, J. M., Rice, P. A. & Steitz, T. A. (1992) *Science* **256**, 1783-1790.
- 15 36. Das, K., Ding, J., Hsiou, Y., Clark, A. D., Jr., Moereels, H., Koymans, L., Andries, K., Pauwels, R., Janssen, P. A., Boyer, P. L. & et al. (1996) *J. Mol. Biol.* **264**, 1085-1100.
37. Huang, H., Chopra, R., Verdine, G. L. & Harrison, S. C. (1998) *Science* **282**, 1669-1675.
- 20 38. Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Jr., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P. & et al. (1993) *Proc. Natl. Acad. Sci. U S A* **90**, 6320-6324.
- 25 39. Becerra, S. P., Kumar, A., Lewis, M. S., Widen, S. G., Abbotts, J., Karawya, E. M., Hughes, S. H., Shiloach, J. & Wilson, S. H. (1991) *Biochemistry* **30**, 11707-11719.
40. Wang, J., Smerdon, S. J., Jager, J., Kohlstaedt, L.

50. Romero, D. L., Busso, M., Tan, C. K., Reusser, F., Palmer, J. R., Poppe, S. M., Aristoff, P. A., Downey, K. M., So, A. G., Resnick, L. & et al. (1991) *Proc. Natl. Acad. Sci. U S A* **88**, 8806-8810.
- 5 51. Young, S. D., Britcher, S. F., Tran, L. O., Payne, L. S., Lumma, W. C., Lyle, T. A., Huff, J. R., Anderson, P. S., Olsen, D. B., Carroll, S. S. & et al. (1995) *Antimicrob. Agents Chemother.* **39**, 2602-2605.
52. Tachedjian, G., Aronson, H. E. & Goff, S. P. (2000)
10 *Proc. Natl. Acad. Sci. U S A* **97**, 6334-6339.
53. Crabtree, G. R. & Schreiber, S. L. (1996) *Trends Biochem. Sci.* **21**, 418-422.
54. Balzarini, J., Brouwer, W. G., Felauer, E. E., De Clercq, E. & Karlsson, A. (1995) *Antiviral Res.* **27**,
15 219-236.
55. Balzarini, J., Brouwer, W. G., Dao, D. C., Osika, E. M. & De Clercq, E. (1996) *Antimicrob. Agents Chemother.* **40**, 1454-1466.
56. Dueweke, T. J., Poppe, S. M., Romero, D. L., Swaney,
20 S. M., So, A. G., Downey, K. M., Althaus, I. W., Reusser, F., Busso, M., Resnick, L. & et al. (1993) *Antimicrob. Agents Chemother.* **37**, 1127-1131.
57. Kleim, J. P., Bender, R., Kirsch, R., Meichsner, C., Paessens, A., Rosner, M., Rubsamen-Waigmann, H.,
25 Kaiser, R., Wichers, M., Schneweis, K. E. & et al. (1995) *Antimicrob. Agents Chemother.* **39**, 2253-2257.
58. Ho, W., Kukla, M. J., Breslin, H. J., Ludovici, D. W., Grous, P. P., Diamond, C. J., Miranda, M., Rodgers, J. D., Ho, C. Y., De Clercq, E. & et al. (1995) *J. Med.*

- Chem.* **38**, 794-802.
59. Pauwels, R., Andries, K., Debyser, Z., Van Daele, P., Schols, D., Stoffels, P., De Vreese, K., Woestenborghs, R., Vandamme, A. M., Janssen, C. G. & et al. (1993) *Proc. Natl. Acad. Sci. U S A* **90**, 1711-1715.
 60. Kuchin, S., Treich, I. & Carlson, M. (2000) *Proc. Natl. Acad. Sci. U S A* **97**, 7916-7920.
 61. Szilvay, A. M., Nornes, S., Haugan, I. R., Olsen, L., Prasad, V. R., Endresen, C., Goff, S. P. & Helland, D. E. (1992) *J. Acquir. Immune Defic. Syndr.* **5**, 647-657.
 62. Ghosh, M., Jacques, P. S., Rodgers, D. W., Ottman, M., Darlix, J. L. & Le Grice, S. F. (1996) *Biochemistry* **35**, 8553-8562.
 - 15 63. Esnouf, R. M., Stuart, D. I., De Clercq, E., Schwartz, E. & Balzarini, J. (1997) *Biochem. Biophys. Res. Commun.* **234**, 458-464.
 64. Barnard, J., Borkow, G. & Parniak, M. A. (1997) *Biochemistry* **36**, 7786-7792.
 - 20 65. Maga, G., Ubiali, D., Salvetti, R., Pregnolato, M. & Spadari, S. (2000) *Antimicrob. Agents Chemother.* **44**, 1186-1194.
 66. Arnold, E. & Rossmann, M. G. (1990) *J. Mol. Biol.* **211**, 763-801.
 - 25 67. Lee, B. & Richards, F. M. (1971) *J. Mol. Biol.* **55**, 379-400.
 68. Ren, J., Milton, J., Weaver, K. L., Short, S. A., Stuart, D. I. & Stammers, D. K. (2000) *Structure Fold Des.* **8**, 1089-1094.

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69. Esnouf, R. M., Ren, J., Hopkins, A. L., Ross, C. K., Jones, E. Y., Stammers, D. K. & Stuart, D. I. (1997) *Proc. Natl. Acad. Sci. U S A* **94**, 3984-3989.